MOLECULAR DETERMINATION OF DIATOM COMMUNITY DYNAMICS IN ONSLOW BAY, NC, AND MONTEREY BAY, CA

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A Thesis Submitted to the
University of North Carolina Wilmington in Partial Fulfillment
Of the Requirements for the Degree of
Master of Science

Department of Biology and Marine Biology

University of North Carolina Wilmington

2007

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ABSTRACT

Diatoms are one of the major primary producers supporting food webs in the ocean. Their productivity could be linked to community composition. Temporal and spatial variation of diatom communities may correlate with nitrogen sources and availability. In order to understand the changes in diatom communities related to environmental variations, molecular tools targeting nitrate transporter genes in diatoms were developed and applied to examine diatom communities in Onslow Bay, North Carolina, and Monterey Bay, California. Cloning, sequencing and Distance-Based OTU and Richness (DOTUR) analyses were used to investigate patterns of diversity over time and space. Terminal Restriction Fragment Length Polymorphism (T-RFLP), Cannonical Correspondence Analysis (CCA) and simple linear regression analyses were conducted to compare community dynamics to variations in environmental parameters. In Onslow Bay, the composition of diatom communities was highly varied in different seasons and locations. Temperature differences appeared to influence the changes in diatom communities more than dissolved inorganic nitrogen. In Monterey Bay, temporal variation was higher in diatom communities as compared to depth profiles. Changes in temperature, silica, and nitrate appeared to influence the composition of diatom assemblages. This study demonstrated the potential usage of molecular tools to monitor diatom communities and to determine the environmental factors affecting the composition of diatom assemblages in nature. This will help to gain a better understanding of phytoplankton community dynamics in the oceans.

ACKNOWLEDGMENTS

My thanks go to Dr. Ken Shull who introduced me to molecular biology and encouraged me to pursue graduate studies in this field. I am grateful to Dr. Bongkeun Song for sponsoring my graduate studies, patiently explaining the infinite complexities of microbiology to a neophyte, and allowing me to pursue the ecological biology that I find most fascinating.

Words cannot express my gratitude to Kim Mayer for her guidance and mentoring. My graduate studies would have been much less enjoyable without her presence. My appreciation goes to Oliva Dale, Kristi Sommer, Holly Oates, Jen Bagwell, and Brian Shirey for the fun and enjoyment my fellow graduate students provided.

Special thanks go to my parents who supported me throughout my studies. Without their love and concern I would not have made it to graduation. Special thanks also go to Pastor Chris Schansberg, the Gordon Wright family, and the congregation of Masonboro Baptist Church for their prayers and support during my graduate studies.

The Department of Biology and Marine Biology, the Graduate School, and the National Science Foundation provided financial support for my research and studies. Thanks go to the Coastal Ocean Research and Monitoring Program for providing water samples and environmental data and Dr. Stephen Brewer for his teaching and assistance with data analysis.

Finally, my thanks go my committee – Dr. Wilson Freshwater and Dr. Carm Tomas – for their guidance, teaching, and assistance throughout my studies.

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CHAPTER 1: INTRODUCTION

DIATOM COMMUNITES IN THE OCEAN

Phytoplankton has a significant role in the global cycles of carbon and nitrogen Oceanic phytoplankton fixes 48.5 petagrams C in primary production (1 Pg = 10^{15} g), nearly 50% of the global carbon fixation (Field et al. 1998), and terrestrial rain forests carry the other half (Parker and Armbrust 2005). Most of the oceanic primary production is found in three distinct bands: between 60 N and 30 N latitude, near the equator, and between 30 S and 60 S latitude. The highest oceanic net primary production occurs in the southern mid-temperate latitudes (Field et al. 1998).

Diatoms make up a large majority of phytoplankton communities. Diatoms composed 20-50% of the phytoplankton community in Chesapeake Bay (Lomas and Gilbert 1999) and 50% of the phytoplankton community in Monterey Bay (Kudela and Dugdale 2000). They also contributed more than 60% of the planktonic biomass measured off the North Carolina coast (Redalje et al. 2002). Diatom communities therefore have significant roles in marine primary production.

Diatom community structures and their contribution to primary production are influenced by a variety of factors. Physical parameters such as light and temperature could significantly affect diatom assemblages. Temperature effects on diatom growth and productivity were observed on the Scotian Shelf as measured by the proportion of fucoxanthin to total chlorophyll-*a* concentration. Fucoxanthin, an indicative pigment of diatoms, increased with decreasing temperature (Bouman et al. 2005). Diatoms generally dominate the phytoplankton assemblage in cool waters, and their physiology is affected

by changing temperatures (Lomas and Glibert 1999). Global warming has been hypothesized as a reason for an observed decrease in large phytoplankton like diatoms in the North Pacific Subtropical Gyre off Hawaii (Karl et al. 2001).

Stratification of the water column has severe effects on diatom communities. Changes in abundance and composition of diatom assemblages were correlated to variations in water column stratification and depth of the oxygen minimum zone off the coast of Chile (Herrera and Escribano 2006). Diatoms were more abundant when the water column was mixed, but smaller dinoflagellates and flagellates were prevalent in more stratified waters. Depth of the oxygen minimum zone may influence community structure indirectly by altering nutrients or by affecting grazing pressures (Herrera and Escribano 2006). Studies on freshwater diatoms in the rivers of Spain showed a change in community structure correlated to water quality factors as well as physiographical factors, including temperature, altitude, and stream order (Herrera and Escribano 2006).

Diatom communities are also influenced by available nutrients, especially nitrogen (Kudela and Dugdale 2000). Nitrogen is one of the major limiting nutrients in the ocean. Nitrogen enrichment experiments conducted in the early 1970s revealed its significance in diatom growth (Ryther and Dunston 1971). Biomass increase of diatoms in coastal water samples was positively correlated with an increase in NO_3/NO_2 concentrations (Redalje et al. 2002). Diatom biomass increased 1.5 to 7-fold when natural phytoplankton assemblages from Long Island Sound, NY, were incubated with nitrogen amendments (Gobler et al. 2006). An increase of diatom biomass was observed in nitrogen enrichment experiments conducted with water samples from Pamlico Sound

in NC (Piehler et al. 2004). Therefore, nitrogen is a significant nutrient for the growth of diatoms and other phytoplankton.

In addition, silica, an important nutrient for diatom shell formation, influences diatom communities. Silicon is required for diatom proliferation (Lewin 1962). Mesocosm experiments conducted with environmental samples from Norway showed a dominance of diatoms when silicate concentration exceeded 2 μM regardless of temperature or nitrate levels (Egge and Aksnes 1992). Silicon regulates gene expression in diatoms (Hildebrand et al. 1993) and appeared to control nitrate uptake rates measured in mesocosm experiments of natural phytoplankton assemblages consisting of 50% diatoms (Kudela and Dugdale 2000). Higher concentrations of silica increased nitrate uptake rates, but silica levels did not directly control biomass production.

Diatoms have a high affinity for taking up nitrate even where the concentrations are less than 40 μmol N L^{-1} (Lomas and Glibert 2000), which is often the upper limit in open ocean systems. The supply of nitrogen from terrestrial sources or seasonal upwelling could initiate diatom blooms. Large chain-forming diatoms are more efficient nitrogen uptake (Herrera and Escribano 2006) than smaller picoplankton, which are frequently found in warmer and nitrogen poor waters (Agawin et al. 2000).

Different nitrogen species also affect the composition of the dominant diatom population. Semeneh et al. (1998) found that centric diatoms dominated in the nitrate-rich open oceanic zone, but and pennate diatoms were abundant in ammonium-rich shallow coasts. The growth rate of the diatom *Thalassiosira pseudonana* was 12% lower when grown using nitrate as the nitrogen source than when using ammonium (Levasseur et al. 1993). In contrast, the diatom *Chaetoceros gracilis* grew faster on nitrate than on

ammonia (Levasseur et al. 1993). Growth on urea was slower for both diatom species when compared to growth on ammonia (Levasseur et al. 1993). The sources and levels of nitrogen could therefore control diatom community dynamics in the ocean.

GENETIC CHARACTERISTICS OF NITROGEN UPTAKE AND ASSIMILATION IN DIATOMS

The pathway of nitrogen uptake and assimilation in marine phytoplankton is well understood (Conway 1977). Nitrate is transported across the cell membrane by a high-affinity or low-affinity nitrate transporter (NRT). High affinity systems operate when external nitrate concentrations are less than 250μM, while low affinity systems operate when external nitrate concentrations are greater than 1mM (Conway 1977). Nitrate is reduced to nitrite by the assimilatory nitrate reductase enzyme (NR) in the cytosol. Nitrite is transported into the chloroplast by a nitrite transporter where it is reduced to ammonium and incorporated into amino acids by glutamate synthase (Galvan and Fernandez 2001). The reduction of nitrate to nitrite is considered the rate limiting step in nitrate assimilation (Allen et al. 2005, Campbell 1999, Berges and Harrison 1995) though it is probable that there is more than one rate limiting step in the nitrogen assimilation pathway (Berges 1997).

Nitrate uptake rates depend on various factors including the type of nitrate transporter (high affinity or low affinity) and the level of nitrate in the environment. Half-saturation constants (K_s) of diatoms vary from 0.4 to 53 μM-N L^{-1} in external nitrate concentrations of 0.4 to 10 μM-N L^{-1} (Collos et al. 2005). Nitrate uptake kinetics are

saturating below concentrations of 40μM-N and non-saturating at concentrations up to 180μM-N (Lomas and Gilbert 1999, Lomas and Glibert 2000). Below concentrations of 40 μM-N, the high-affinity nitrate transport system is in effect, but the high-affinity system becomes saturated, meaning uptake rates stop increasing, at 40 μM-N. Over 40 μM-N the low-affinity nitrate transport system takes over and nitrate uptake rates increase in a linear fashion with no detected saturation point (Lomas and Glibert 2000). As nitrate concentration increases uptake rate increases in diatoms (Lomas and Glibert 2000, Collos et al. 2005).

Nitrate transporters have a specific intramembrane structure of 12 transmembrane domains arranged in two sets of six interconnected by a cytosolic loop (Galvan and Fernandez 2001) and are responsible for the active transport of nitrate across the cell membrane (Falkowski 1975). There have been 52 different genes in plants considered to be involved in nitrate transport. They are classified into two families: NRT1, low-affinity transporters, and NRT2, high-affinity transporters (Galvan and Fernandez 2001, Forde 2000, Crawford and Glass 1998, Unkles et al. 2004). Two specific amino acids, R87 and R368, must be present in the protein for it to function properly, and these two amino acids likely act in substrate binding in high-affinity nitrate transporters (Unkles et al. 2004).

Regulation of nitrate transporters is less well understood due to its complexity. Nitrate transporters are inhibited 20 to 60% by ammonium and induced by nitrate (Conway 1977, Galvan and Fernandez 2001), though ammonium inhibition appears not to be as intense as once considered (Dortch 1990). Nitrate uptake is decoupled from nitrate assimilation (Smith et al. 1992) based on the observation of an intracellular

accumulation of nitrate. This nitrate pool may be used in times of low available nitrogen. The nitrate pool is also used to remove excess electrons under high light and low temperature conditions (Lomas and Gilbert 1999, Parker and Armbrust 2005).

Diatom nitrate transporter genes were first detected from a pennate diatom *Cylindrotheca fusiformis* (Hildebrand and Dahlin 2000), which were phylogenetically distinct from those found in higher plants and green algae. Additional NRT2:1 diatom nitrate transporter genes were detected using PCR amplification with specific primers for diatoms as designed by Song and Ward (2007). Sequence analysis of the nitrate transporter genes showed that the phylogenies of the genes were congruent with taxonomic classification of the examined diatom species (Song and Ward 2007). Nitrate transporter genes could therefore be used as a genetic marker to detect and monitor diatom communities in natural ecosystems.

T-RFLP ANALYSIS OF COMMUNTIY STRUCTURE

Various molecular techniques have been used to detect and analyze community structure and dynamics of microorganisms in the environment. PCR amplification and subsequent cloning and sequencing provide phylogenetic information about microbes based on the targeted genes. However, cloning and sequencing require substantial efforts to examine the variation in microbial community. Alternative fingerprinting approaches such as Terminal Restriction Fragment Length Polymorphism (T-RFLP) and Denaturing Gradient Gel Electrophoresis (DGGE) are rapid and sensitive molecular tools used to

track changes in community composition and comparative community analysis (Marsh 1999).

In T-RFLP analysis, PCR amplification of a target gene is conducted with one fluorescently labeled primer and one non-labeled primer. The PCR products are then digested with a restriction endonuclease, resulting in different sized fragments due to sequence differences of the genes in different microbes. The digested fragments are separated by gel electrophoresis and the sizes of the fragments are plotted on a chromatograph of peak profiles. Differences in peak profiles among samples can be interpreted as variations in community structure.

This technique is most commonly used for 16S rRNA genes because the genes are universal and can target an entire bacterial community (Marsh 1999), but functional genes can be targeted for T-RFLP analysis to examine community structure and dynamics of specific functional groups. For instance, Braker et al. (2000) and Braker et al. (2001) used the nitrite reductase genes *nirS* and *nirK* to investigate the denitrifying community in Pacific Northwest marine sediments. Thus, the genes encoding NRT2 in diatoms can be a good genetic candidate to detect and monitor diatom community dynamics in natural ecosystems.

PURPOSE OF THE CURRENT STUDY

Diatom communities vary over space and time in Onslow Bay and Monterey Bay, which may be correlated to nitrogen source and availability. Changes in the diatom community can be monitored by using molecular tools targeting the nitrate transporter

genes specific for diatoms. I hypothesize that changes in diatom communities as indicated by changes in nitrate transporter genes will be strongly correlated to changes in nitrogen source and availability. The diatom communities in Monterey Bay will be influenced by different environmental factors from those in Onslow Bay. Specifically, Monterey Bay diatom communities will have a stronger correlation to nitrate levels than Onslow Bay diatom communities due to regular pulses of high nitrate from seasonal upwelling events in Monterey Bay.

CHAPTER 2: MONITORING DIATOM COMMUNITY DYNAMICS IN ONSLOW BAY, NC, USING NITRATE TRANSPORTER GENE AS A GENETIC MARKER

INTRODUCTION

Onslow Bay, located between Cape Lookout and Cape Fear off the coast of North Carolina, comprises the northern region of the South Atlantic Bight (SAB). The SAB is located between Cape Hatteras in North Carolina and Cape Canaveral in Florida, and its nearshore areas contain very active shellfish and finfish fisheries. Onslow Bay has a unique food chain structure, which seems to be driven by benthic microalgae instead of the pelagic phytoplankton that is dominant in other regions of the SAB (Mallin et al. 2005). Diatoms composed of 90% of the benthic communities in Onslow Bay (Cahoon and Laws 1993), and more than 60% of the biomass measured off the North Carolina coast (Redalje et al. 2002). Benthic diatoms, including *Cocconeis* spp, *Amphora* spp., and *Navicula* spp, were found to compose the basis of the Onslow Bay food chain (Cahoon and Laws 1993). Hence diatom communities have significant roles in primary production in Onslow Bay and the SAB.

Diatoms are influenced by available nutrients, especially nitrogen and silica (Kudela and Dugdale 2000). The increase of diatom biomass measured off the coast of North Carolina was positively correlated with NO_3^-+NO_2^- concentrations (Redalje et al. 2002). Diatom biomass increased 1.5 to 7-fold when natural assemblages from the Long Island Sound, NY, were incubated with nitrogen addition (Gobler et al. 2006). The increases of diatom biomass (12-26 %) were also observed in nitrogen amendment experiments with samples from the Pamlico Sound, NC (Piehler et al. 2004). Silica is an

important nutrient for diatom growth due to the formation of a silica shell around diatom cells. In mesocosm experiments in which natural phytoplankton assemblages consisting of 50% diatoms were enriched with both silica and nitrate, nitrate uptake rates were influenced by silica levels. The concentration of silica adjusted the rate at which nitrogen limitation occurred, but silica levels did not directly control biomass accumulation (Kudela and Dugdale 2000).

Physical parameters such as light and temperature could also be controlling factors of diatom communities. Primary production measured at Cape Hatteras, North Carolina, just north of Onslow Bay, was positively correlated with temperature and light (Redalje et al. 2002). Phytoplankton productivity has been shown to increase with warmer water temperatures in the summer and decrease when the water cools in North Carolina waters, suggesting a correlation with temperature (Mallin et al. 2000).

Nitrogen in Onslow Bay and the SAB is supplied by a variety of sources. Ammonium is the dominant form of nitrogen in Onslow Bay, though there is a constant low level presence of nitrate (Mallin et al. 2005). Intrusions of Gulf Stream water bring nitrogen to Onslow Bay and eddies can force the nutrient-laden water all the way into the estuaries in the SAB (Mallin et al. 2000, Lohrenz et al. 2002). Mixing driven by wind is the primary method by which nutrients trapped near the bottom are brought into the photic zone; only rarely does upwelling occur in the SAB as a result of extreme Gulf Stream intrusions into the SAB (Flagg et al. 2002). Therefore the nitrogen regime changes throughout the year in Onslow Bay as well as the temperature regime, which ranges from 9^{o}C in winter to 27^{o}C in summer (CORMP website (http://www.cormp.org/indexreal.php). These differences in nutrients and temperatures

may correlate with diatom community variation and make Onslow Bay an excellent area for study to understand the effects of both parameters.

The objectives of this study were to examine the composition and dynamics of diatom communities in Onslow Bay, NC over space and time using the nitrate transporter gene (NRT2:1) and to investigate potential links between environmental parameters and diatom community variation. The sequences and T-RFLP profiles of the NRT2:1 genes were compared to variations in temperature, nitrate, and ammonium levels to elucidate correlations between the environmental parameters and diatom community variation.

MATERIALS AND METHODS

Samples and DNA Extraction.

Water samples were collected by CORMP (Coastal Ocean Research and Management Program, University of North Carolina at Wilmington) from three sites in Onslow Bay, NC: OSB5, approximately 15 m deep and located 8 km offshore; OSB15, approximately 23 m deep and located 24 km from shore; and OSB27, approximately 30 m deep and located 45 km from shore (Figure 1). Samples of both surface (S) and bottom (B) water were collected from each site during three different cruises in 2005 - January (J), May (M), and August (A) - for a total of eighteen samples. At each sampling time, temperature and salinity were measured using a SBE-25 CTD Rosette Water Carousel. The levels of nitrate and ammonium were measured using a Bran and Luebbe AutoAnalyzer 3 after bringing the samples into the laboratory. Approximately four liters

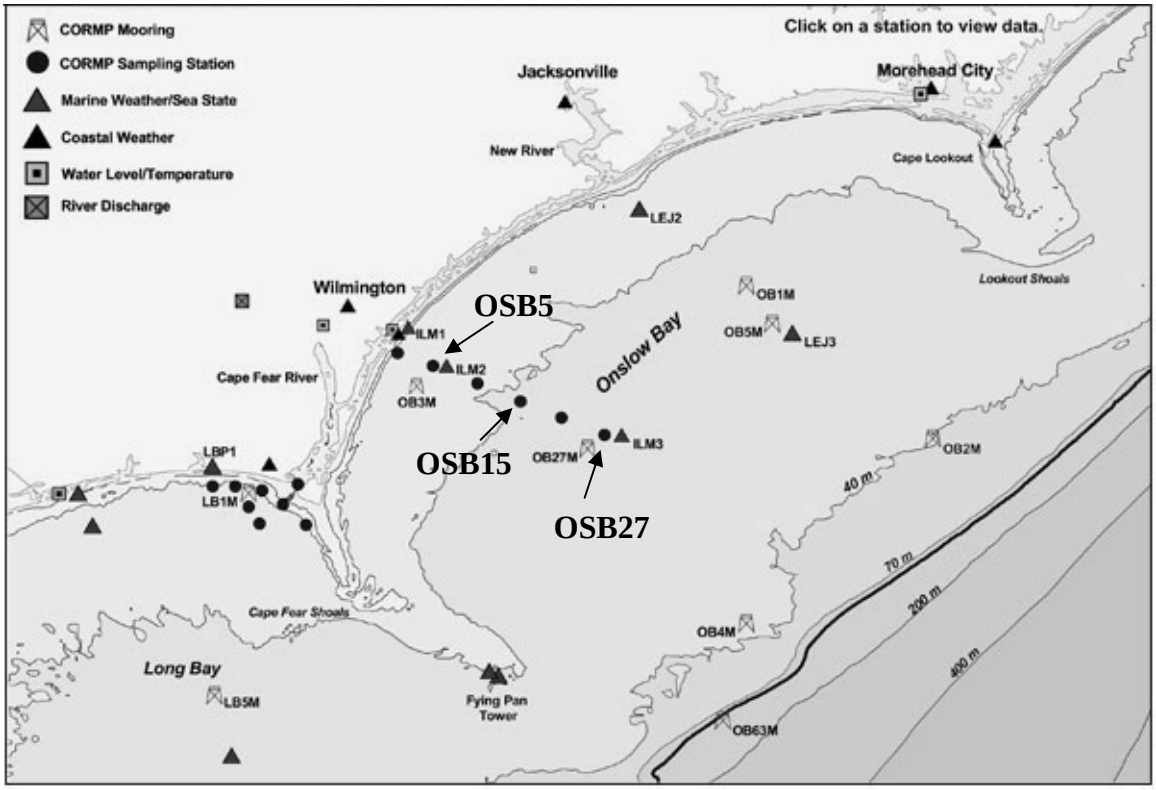

Figure 1. Map of Onslow Bay, NC, including sampling sites used in this study.

from each sample were filtered through a Millipore filter (0.45 μm pore size) to collect the biomass, and filters were stored at -80^{o}C until processing. Temperature, ammonium, and nitrate data (Table 1) for each sample were obtained from the CORMP website (http://www.cormp.org/indexreal.php).

DNA was extracted from the filters using a PowerSoil DNA Isolation Kit (MoBio Laboratory, Carlsbad, CA). The filter for each sample was ground fine in the bead tube with a sterile pestle and the resulting slurry was used in the extraction according to the manufacturer's instructions. Extracted DNA was stored at -20^{o}C until analysis.

PCR Amplification of Nitrate Transporter Genes

In order to examine diatom community structure in OSB, high affinity nitrate transporter (NRT2:1) genes were amplified with specific primers designed by Song and Ward (2007) (Table 2). Nested PCR amplification using the primers DANAT1F and DANAT1R in the first reaction and the primers DANAT3F and DANAT2R in the second reaction was conducted with DNA extracted from samples collected from OSB27 Surface January (SJ), OSB27 Bottom January (BJ), OSB27 Surface May (SM), and OSB27 Bottom May (BM). *Skeletonema costatum* was used as a positive control. In both reactions the 25 μL reaction mix contained 1 μL template DNA, 200 nM each primer, 0.5 μL *Taq* polymerase, 50 μM each deoxynucleotide triphosphate, 2 mM $MgCl_2$, and buffer (500mM KCl, 200mM Tris-HCl [ph 8.4]). The template for the initial PCR reaction was DNA extracted from the environment, and the PCR mixture of the initial reaction was used as a template for the second reaction. The PCR cycle started with an initial

Table 1. Environmental parameters measured at three stations in Onslow Bay, NC.

Name	Temp (^{o}C)	NO_3 (μM)	NH_4 (μM)
OSB5SJ	9.771	1.7	0.825
OSB5BJ	9.762	6.097	0.739
OSB5SM	18.532	0.572	1.168
OSB5BM	16.852	0.107	0.993
OSB5SA	27.898	0.41	0.419
OSB5BA	27.919	0.459	0.316
OSB15SJ	11.688	0.217	1.141
OSB15BJ	11.659	0.146	1.133
OSB15SM	19.290	0.258	1.177
OSB15BM	16.852	0.307	1.102
OSB15SA	28.166	0.237	0.461
OSB15BA	27.919	0.449	0.459
OSB27SJ	13.118	1.852	0.997
OSB27BJ	13.128	0.561	0.785
OSB27SM	19.730	0.081	1.159
OSB27BM	16.977	4.403	0.974
OSB27SA	28.355	0.401	0.422
OSB27BA	25.030	0414	0461

Table 2. Diatom specific NRT2:1 gene primers used in this study.

Primer	Orientation	Sequence (3'– 5')	Tm (^{o}C)
DANAT1F	Forward	CCAAGCCCCACATGAGGRCNTTYCANYT	74.6
DANAT1R	Reverse	GGACAGAGCGCCGGTRTTNCCNCC	69.1
DANAT3F	Forward	GCTGCACCTTCGTGATGTGYCARTAYT	64.4
DANAT2R	Reverse	GGTGGAGCCCTCNGCNGCYTG	67.1

denaturation at 95°C for 10 minutes, and 30 cycles of PCR were as follows: 30 seconds at 95°C, 30 seconds at 55°C, 1 minute at 72°C. After the last cycle, the reaction was extended at 72°C for 10 minutes. PCR products were verified by using electrophoresis with 1% agarose gel.

Cloning and Sequencing.

The nested PCR products were gene cleaned using the PerfectPrep Gel Clean Up Kit (Promega, Madison, WI). A total of 2μL of purified amplicon was used in a cloning reaction using a TOPO TA Cloning® Kit (Invitrogen Corporation, Carlsbad, CA) using half the volumes specified by the manufacturer. The ligated plasmids were transformed in high-transforming-efficiency *Escherichia coli* TOP10® cells (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's protocol and then plated on Lauria Broth agar plates containing 10μg/mL of kanamycin and 100mg/mL of X-gal(5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside). The positive clones carrying the amplified products were determined on the basis of white and blue color screening. The positive clones were picked with sterile toothpicks and grown in LB broth containing10μg/mL of kanamyacin in a 96-well plate. Clonal libraries of NRT2:1 genes were generated in a 96-well plate for each amplified product from OSB27 Surface January (OSB27SJ), OSB27 Bottom January (OSB27BJ), OSB27 Surface May (OSB27SM), and OSB27 Bottom May (OSB27BM).

Ninety-six clones from each library were screened for inserts using colony check PCR with the M13R and T7 primers according to the following protocol: the 25 μL

reaction mix contained 1 μL cloned cells, 200 nM each primer, 0.5 μL *Taq* polymerase, 50 μM each deoxynucleotide triphosphate, 2 mM $MgCl_2$, and 10X PCR buffer (500mM KCl, 200mM Tris-HCl [ph 8.4]). After an initial denaturation at 95°C for 10 minutes, the PCR parameters for 30 cycles were as follows: 30 seconds at 95°C, 30 seconds at 55°C, 1 minute at 72°C. After the last cycle, the reaction was extended at 72°C for 10 minutes. PCR products were then subjected to electrophoresis in 1% agarose gel.

Approximately 25 positive clones from each library were sequenced. Aliquots (1 μL) of products from the colony screening PCR was used for each sequencing reaction, along with either the primers M13R or T7. DNA sequencing was performed with an ABI PRISM BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Samples were initially denatured by heating to a temperature of 96°C at a rate of 1°C/second and then holding at 96°C for 1 minute. After the initial denaturing, the PCR parameters for 25 cycles were as follows: cooling to a temperature of 50°C at the rate of 1°C/second, holding at 50°C for 5 seconds, heating to 60°C at the rate of 1°C/second, holding at 60°C for 4 minutes, heating to 96°C at the rate of 1°C/second, holding at 96°C for 10 seconds. After the last cycle, the samples were held at 50°C for 5 seconds and then 60°C for 4 minutes. An ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA) was used to analyze the samples, and DNA sequences were assembled and edited using the Sequencher program, version 4.0 (Gene Codes Corporation). A neighbor-joining tree was produced from the sequence data using the Molecular Evolutionary Genetics Analysis (MEGA) software version 3.1 (Kumar et al. 2004) using the Kimura 2-parameter model for nucleotide substitutions and 500 bootstrap replicates.

T-RFLP Analysis of NRT2:1 Genes

In order to examine diatom community structures at three OSB stations (5, 15 and 27), T-RFLP analysis of diatom NRT2:1 genes were conducted using a nested PCR amplification as described above using the primers DANAT1F and DANAT1R in the first reaction and the fluorescently-labeled primer DANAT3F (6'-FAM) and DANAT2R in the second reaction. Environmental DNA was extracted from total 18 samples - the surface (S) and bottom (B) water at three stations (OSB5, OSB15 and OSB27) in three different months (January, May and August) - and used for templates for the first PCR reaction. PCR products were subjected to electrophoresis in 1% agarose gel and the band of the correct size excised and extracted using the PerfectPrep Gel Clean Up Kit (Promega, Madison, WI). A total of 10 μL of purified PCR products were digested overnight with 0.5 U of *HaeIII* enzyme in 15 μL volume at 37^{o}C. The digested product was precipitated with 75% isopropanol and resuspended in 10 μL of Hi-Di formamide with GS500 ROX size standard (Applied Biosystems, Foster City, CA) and then denatured for 90 seconds at 95^{o}C and placed immediately on ice for 1 minute. The digested products were run on an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA) in GeneScan mode. T-RF fragments with more than 10% of the height of the largest T-RF were considered distinct from background variation and used in further analysis (Figure 2).

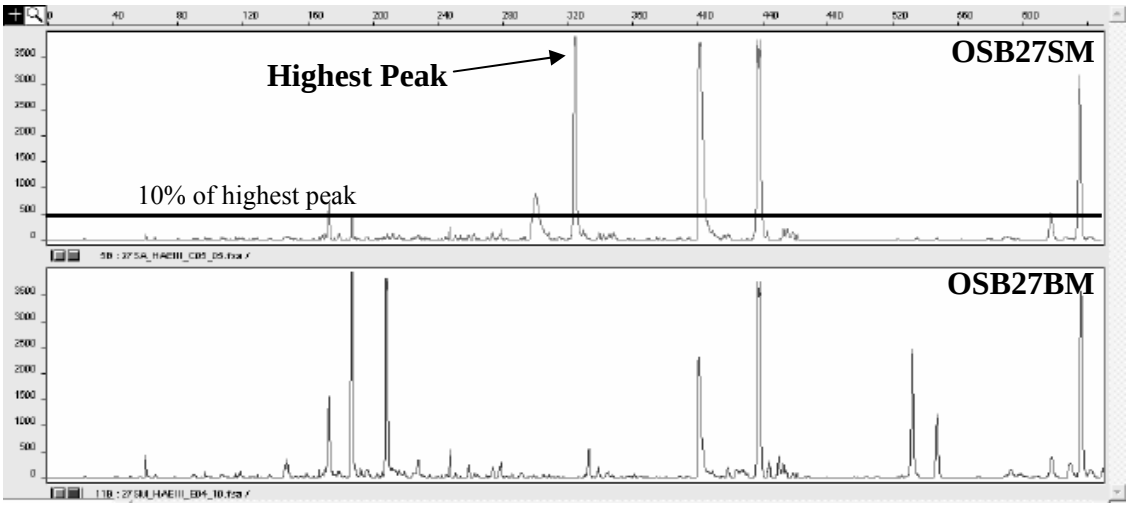

Figure 2. Determination of T-RF cutoff based on height.

Community Structure Analysis

Community structures of diatoms in Onslow Bay were compared with sequences from the NRT2:1 gene libraries and T-RFLP fingerprints. T-RFLP fingerprints obtained from three different stations, three different months and two depths were used for analysis. Pairwise comparisons were conducted for each sample, and a community diversity index (C) for each comparison was calculated using the formula: 1 - (W/(a1+a2)) where W is the number of T-RF peaks samples 1 and 2 have in common, a1 is the total number of peaks in sample 1, and a2 is the total number of peaks in sample 2 (modified from Hewson and Fuhrman 2004). Pairwise comparisons of each environmental parameter (temperature, nitrate and ammonia) for each sample were also conducted by noting the change in each parameter between each sample.

Simple linear regression was performed for the combined data set of 18 samples as well as the individual stations for each environmental parameter using MINITAB statistical software. In addition, Canonical Correspondence Analysis (CCA) was conducted on all samples using the PC-ORD version 4.20 software (McCune and Mefford 1999). Community differences based on the presence or absence of T-RF peaks of given sizes were determined using Jaccard's index for all analysis, and community differences were compared to the variations in temperature, nitrate and ammonium levels. Statistical significance was determined using a Monte Carlo test and Mantel's test.

Sequences of the NRT2:1 genes from the station OSB27 were used to determine the variation in diatom communities using Molecular Evolutionary Genetics Analysis (MEGA) version 3.1 (Kumar et al. 2004). Sequences were aligned in MEGA and

obvious sequence errors were amended. Sequences were designated into four groups based on their clonal libraries: OSB27SJ, OSB27BJ, OSB27SM, and OSB27BM. Net community difference indices were then calculated by a net between group means using the Kimira-2 parameter model. The net difference indices were used to compare differences in environmental parameters and simple linear regression was performed for each environmental parameter using Microsoft Excel.

Diatom community diversity was compared using DOTUR (Distance-Based OTU and Richness) to investigate patterns of diversity in each sample from the station OSB27. A rarefaction curve was produced using DOTUR (Schloss and Handelsman 2005) and a level of 4% differences in sequences were used to determine the operational taxonomic units (OTUs) for the OSB27SJ, BJ, SM, and BM libraries. A Chao[1] estimator and Shannon index were also used to determine diversity of the NRT2:1 genes in Onslow Bay.

RESULTS

PCR Detection of NRT2:1 Gene

A nested PCR was performed on DNA extracted from environmental samples from Onslow Bay using primers specific for diatom nitrate transporter genes (NRT2:1). No PCR product was found from the initial reaction. Nested PCR generated the expected 750 bp product from all samples with the exception of the OSB5SA sample. The OSB5SA sample yielded two PCR fragments of 750 bp and 1000 bp. The products with

750 bp were selected for cloning. Four different libraries (OSB27SJ, BJ, SM and BM) were generated for sequence analysis. The number of sequenced clones from each library is listed in Table 3.

Phylogenetic Analysis of NRT2:1 Genes

Phylogenetic analysis showed all the sequenced clones were more closely related to the NRT2:1 genes found in diatoms than to a green alga *Chlamydomonas reinhardtii* (Figure 3). Most of the sequences were grouped within 10 clusters based on sequence similarities. Cluster A and I contained more sequences from May than January, which might show the increases of diatom population carrying the NRT2:1 genes belonging to clusters A and I during the spring. In contrast, the population carrying the NRT2:1 genes in cluster J decreased in May. Several unique populations of diatoms were present at each sample as shown in cluster B, R, F and G, as well as the clones NT OSB27BM C3, NT OSB27SM H9, NT OSB27BJA6, and NT OSB27SJ E6. Cluster D and H have the NRT2:1 genes found only in May, which represent the unique diatom populations in this month. Cluster G with two sequences from the bottom of May sample were related to the genes found in a pinnate diatom *Cylindrotheca fusiformis* with more than 55 % sequence similarities. Cluster H were associated with *Thalassiosira pseudonana* and *Skeletonema costatum* with more than 50 % similarities. However, most of the NRT2:1 genes found in the OSB27 station were not closely related to the genes found in the reference diatom species. In general, the NRT2:1 genes from January and May segregated as shown in different clusters. The presence of different diatom populations (Clusters B, D, E and F)

Table 3. PCR detection of NRT2:1 genes and number of sequenced clones from Onslow Bay station OSB27 samples.

Sample	DANAT 1F/DANAT 1R	DANAT 3F/DANAT 2R	# of clones
OSB27SJ	-	+	22
OSB27BJ	-	+	23
OSB27SM	-	+	26
OSB27BM	-	+	23

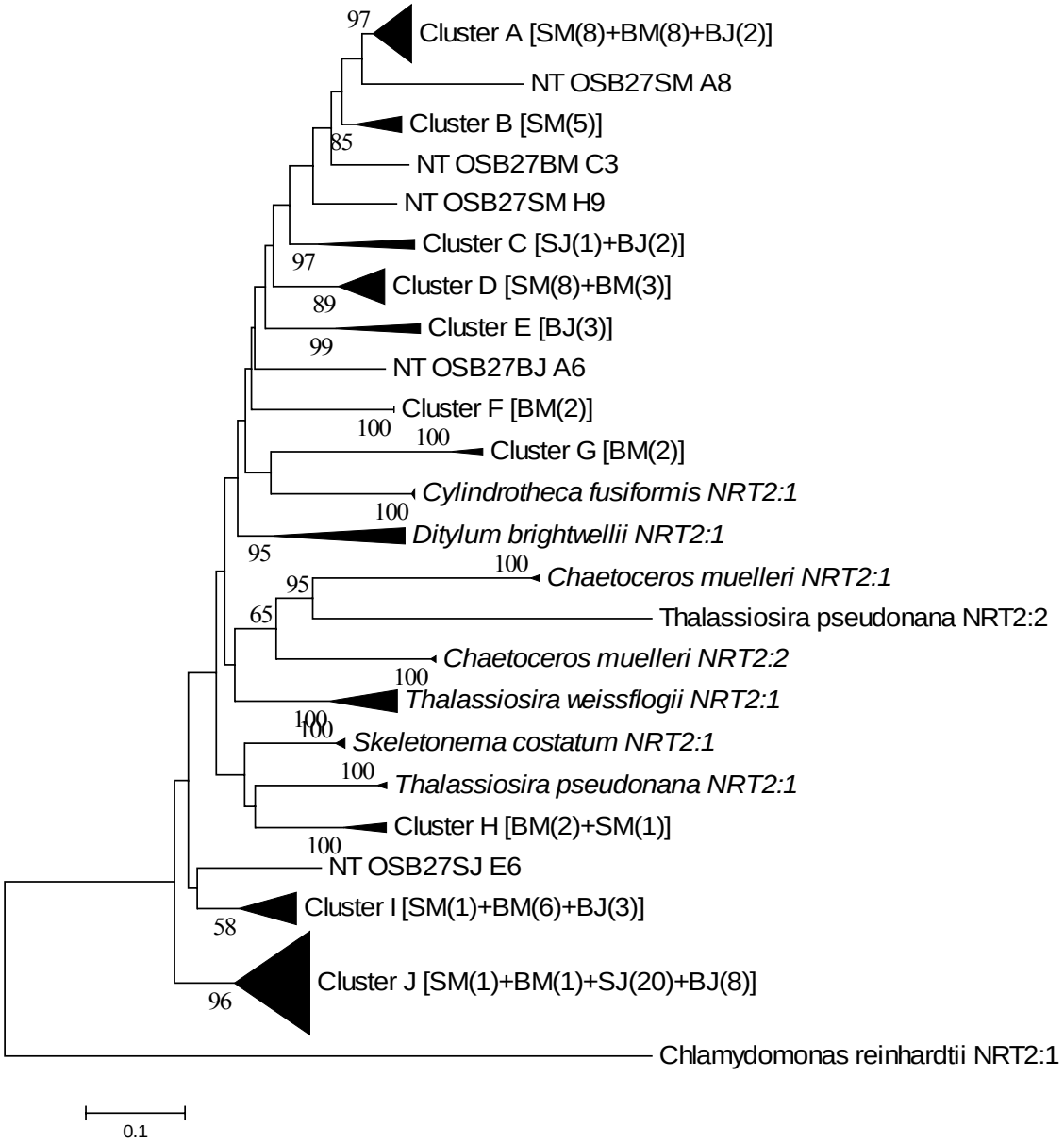

Figure 3. Phylogenetic tree of NRT2:1 genes from station OSB27 in Onslow Bay.

was found in different depths as the samples were collected from the surface and bottom of the station. Thus, seasonal and spatial variation of diatom communities were observed in the OSB27 stations based on the NRT2:1 gene sequences.

Community Structure Analysis Based on NRT2:1 Gene Sequences

MEGA analysis of net community differences using sequence data was compared to environmental parameters to investigate potential links between environmental parameters and diatom community variation (Figure 4). The community differences based on the MEGA analysis ranged from 0.009 to 0.16 (Table 4). The surface January communities and surface May communities were most diverse (0.16), while the surface and bottom communities collected in May were least diverse (0.009). No environmental parameter differences showed a statistically significant relationship to community differences (all R-squared values < 0.23).

Rarefaction analyses of the NRT2:1 genes from the OSB27SJ, BJ, SM, and BM libraries were conducted to compare the diversity of diatom communities in station OSB 27 (Figure 5). Twenty-one OTUs were identified for OSB27SJ, 20 OTUs for OSB27BJ, 16 OTUs for OSB27SM and 17 for OSB27BM using a 4% difference as a definition of a phylotype of the NRT2:1 gene sequences. OTUs represent the number of distinct phylotypes present in a given community.

Phylotype diversity was further examined using the Chao1 and Shannon indices to determine phylotype richness and diversity, respectively. Both indices were calculated using a 4% difference definition of a phylotype (Table 5). The Chao1 index, which is

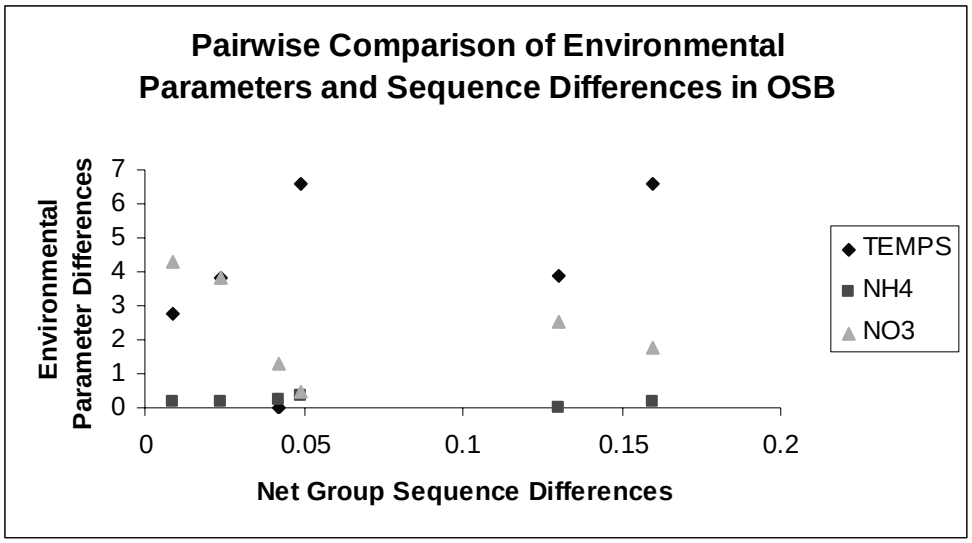

Figure 4. Pairwise comparison between environmental parameters and NRT2:1 gene sequences from station OSB27 in Onslow Bay

Table 4. Net community differences based on MEGA analysis.

	27 SJ	**27 BJ**	**27SM**
27 SJ			
27 BJ	0.042		
27 SM	0.16	0.049	
27 BM	0.13	0.024	0.009

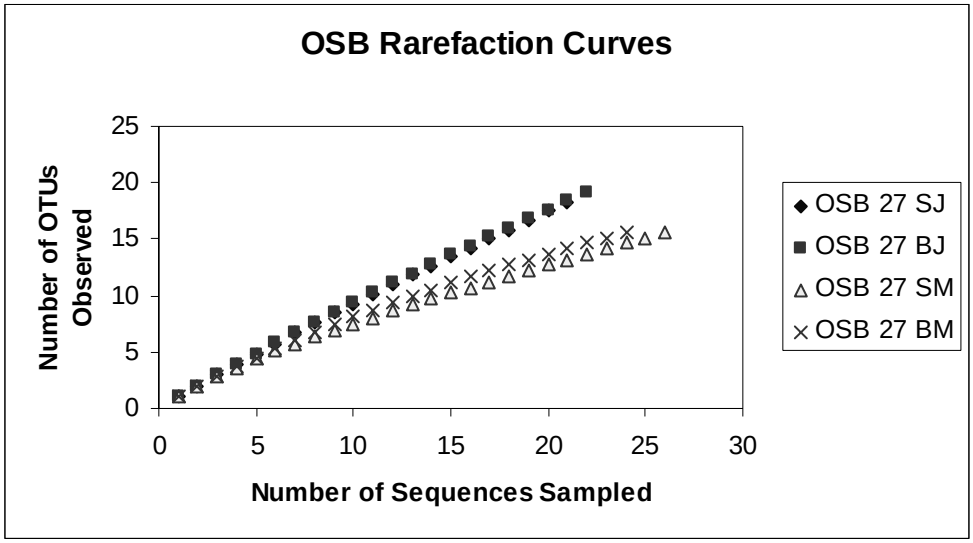

Figure 5. Rarefaction analysis of the OSB27 clone libraries.

Table 5. Chao1 and Shannon indices of diversity for OSB libraries.

Site	Sequence #	Rarefaction #	Chao1 index	Shannon index
OSB27SJ	22	21	87	2.87822
OSB27BJ	23	20	96.5	2.93192
OSB27SM	27	16	38	2.49694
OSB27BM	25	17	29.75	2.5988

a nonparametric estimator, gives a minimum estimation of species richness. January samples have a Chao1 estimate of 87 and 96.5 OTUs, indicating higher phylotype richness than May samples (29.75 and 38 OTUs). The Shannon index is an estimation of species diversity that ranges from 0 to about 4.5, with the higher range representing the most species diversity. The Shannon index also indicates that January samples (2.88 and 2.93) have higher phylotype diversity than May samples (2.50 and 2.60).

T-RFLP Analysis and CCA

Spatial and temporal variation of marine diatom communities in Onslow Bay was observed using T-RFLP analysis of the NRT2:1 genes. T-RF peaks were considered for analysis as separate from the background if their height was higher than 10% of the highest peak. T-RF peak sizes ranged from 60 bp to 636 bp, and each sample contained between 4 and 17 peaks (Figure 6, Figure 7, and Figure 8). None of two fingerprints had the exact same T-RF pattern, but most samples contained T-RFs of the following sizes (bp): 173, 187, 209, 437, and 532. The T-RFs were unable to be identified in the sequenced clones based on *in silico* analysis.

Pairwise comparisons between T-RFLP fingerprints and environmental parameters revealed that only temperature changes had a statistically significant relationship to diatom community differences based on regression analyses ($p=0.002$, Figure 9).

The presence or absence of T-RF peaks in T-RFLP can also be converted into a binary sequence and used in CCA. CCA is a statistical analysis specifically designed to

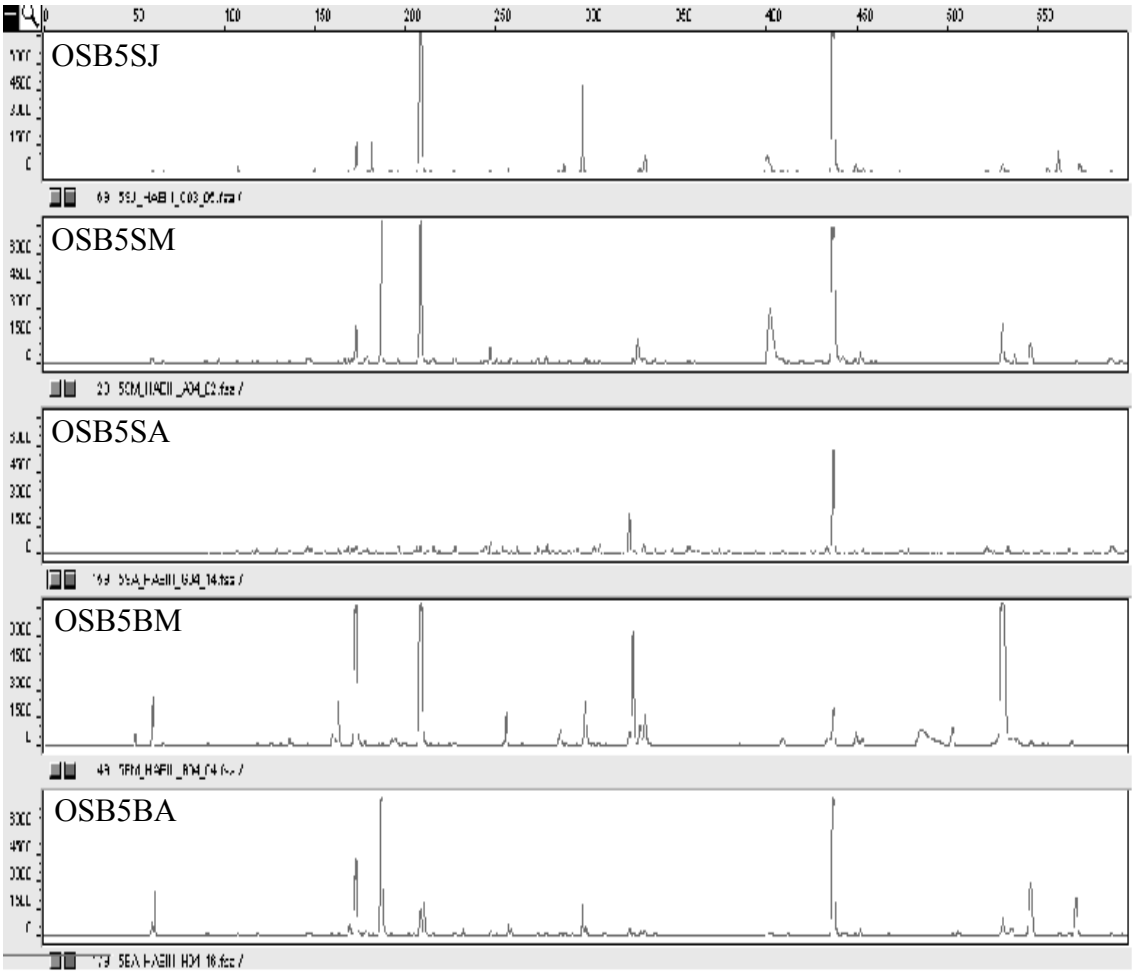

Figure 6. T-RFLP fingerprints from station OSB5.

S: surface, B: bottom,

J: January, M: May, A: August

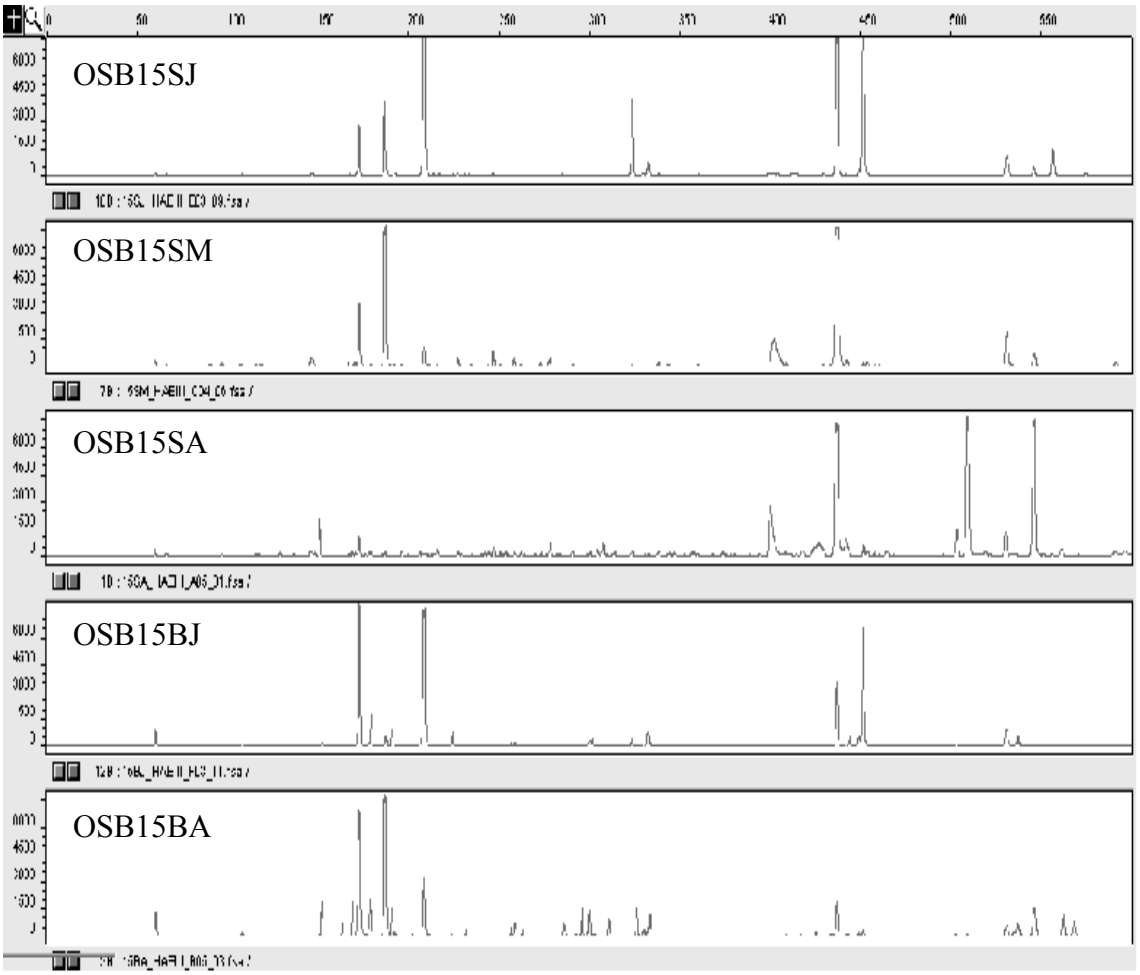

Figure 7. T-RFLP fingerprint profiles from station OSB15.

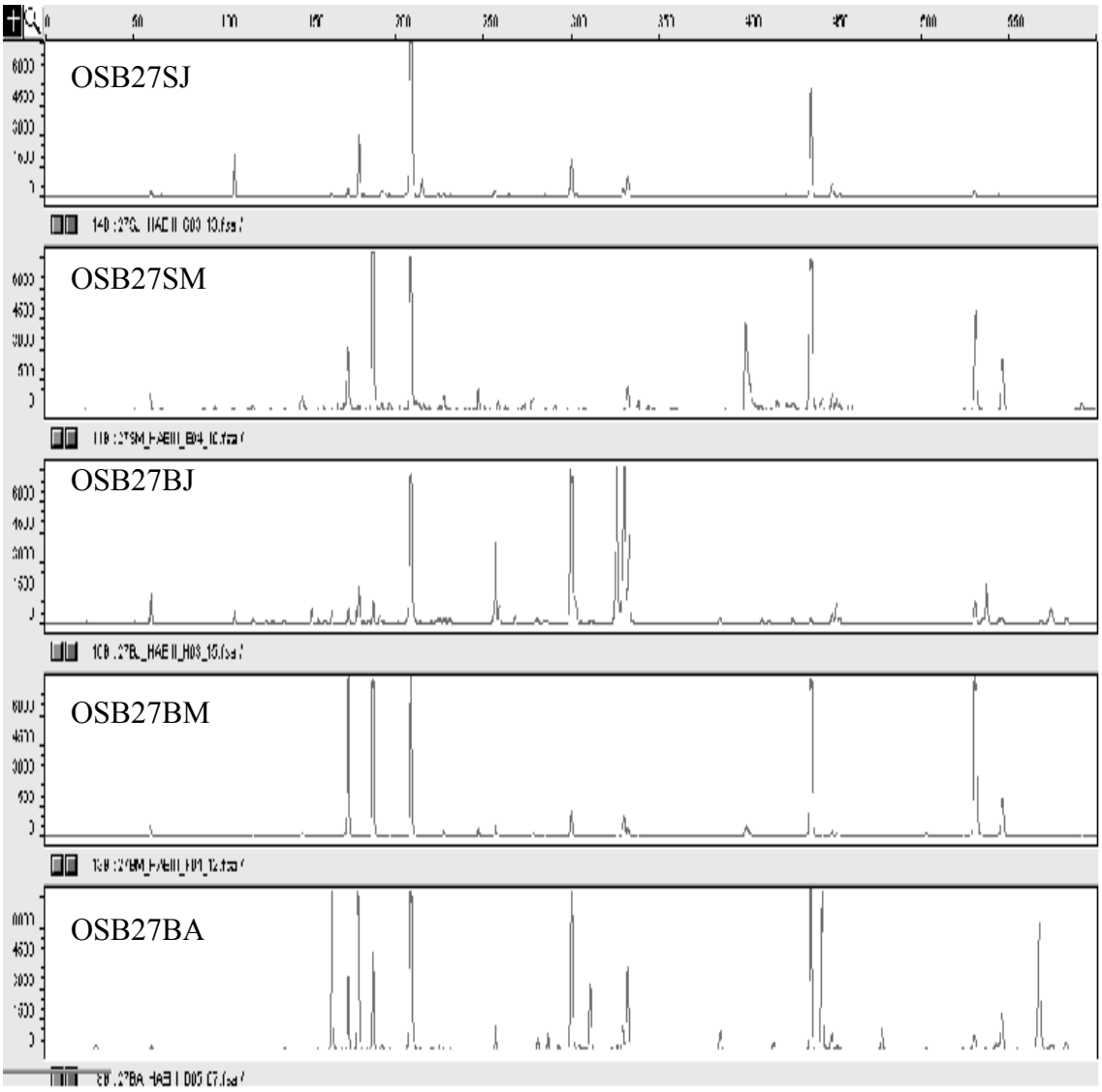

Figure 8. T-RFLP fingerprints from station OSB27.

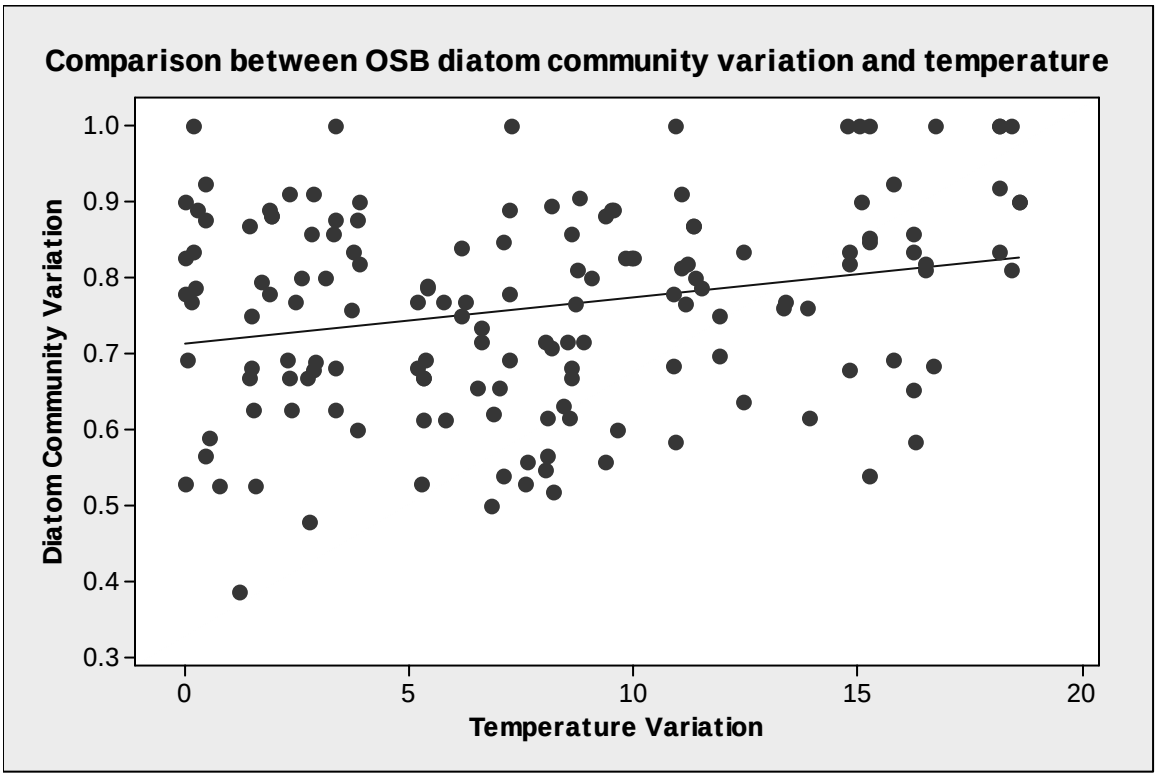

Figure 9. Pairwise comparison between temperature and T-RFLP fingerprints from three stations in Onslow Bay.

relate environmental variation to community variation. CCA showed different trends of correlations between T-RFLP fingerprints and environmental parameters such as nitrate, ammonia, and temperature. Variations of communities in OSB15SM, OSB27SJ, OSB27BM and OSB27SM were highly correlated to differences in nitrate levels. However, the rest of the communities appeared to be more influenced by temperature and levels of ammonium. There was no clear segregation of communities based on different stations or months. The community at station OSB5BJ was the most unique, never grouping with any other sample. Samples from the surface would usually group separately from samples from the bottom along the temperature axis (Figure 10). Mantel's test to determine correlation between two distance matrices was also performed using all OSB T-RFLP samples and environmental data (nitrate, ammonium, and temperature). No correlation was found between the diatom communities and ammonia ($p=0.41$), nitrate ($p=0.46$) or temperature ($p=0.19$), but temperature had the lowest p-value of the three parameters.

DISCUSSION

Phylogenetic analysis of the NRT2:1 genes in the OSB27 station showed a distinct shift in diatom communities over season, but no significant change between different depths. Very few sequences from the January library clustered with the May samples, indicating a shift in the diatom communities from winter to spring. It was hypothesized that changes in diatom communities would be most influenced by changes in nutrient regimes, specifically nitrogen levels. Nitrate and ammonia levels in Onslow

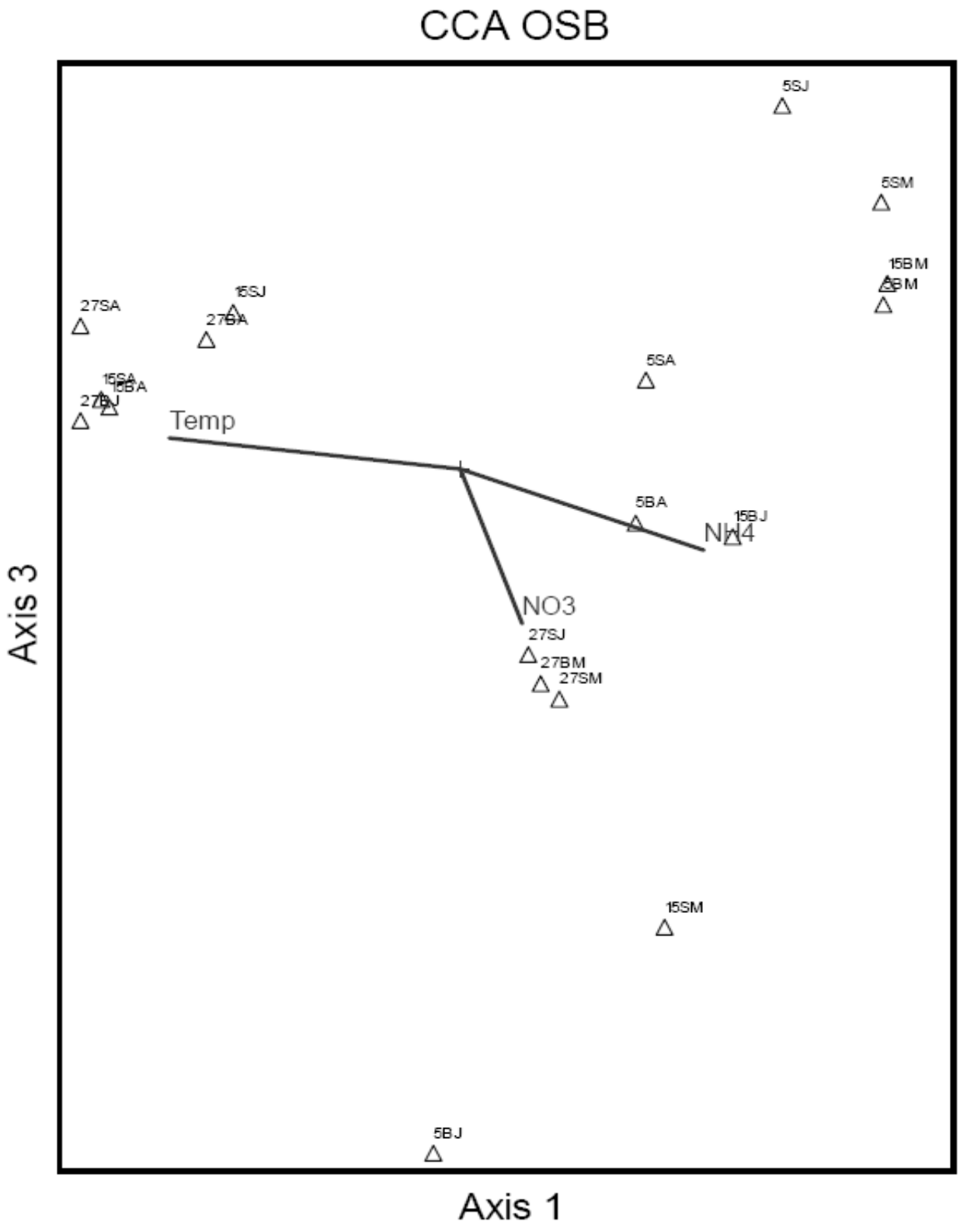

Figure 10. CCA analysis of T-RFLP fingerprints and environmental parameters.

Bay, NC, are similarly low throughout the water column, so there is little differentiation between depths based on the NRT2:1 gene sequence analysis. However, there is a change in the nitrate and ammonium levels from winter to spring as well as in temperature, and distinct clusters form in the phylogenetic tree according to season. This pattern of clustering may reflect the changes in nutrient or temperature levels from winter to spring.

DOTUR analysis was used to examine the diversity of the diatom communities from OSB27 over different times and depths. Operational taxonomic units (OTUs) are groups of sequences considered to be one phylotype as defined by a certain percentage sequence similarity. A 3% sequence similarity has been the generally accepted cutoff for previous ecological studies, but the software would not produce data at the 3% cutoff for every OSB27 library. A 4% sequence similarity was chosen as the cutoff in this study because this was the closest percentage the software would generate for all samples to the generally accepted standard. Phylotype diversity and richness were the greatest in the winter season at station OSB27 according to the Chao1 and Shannon indices.

The net community differences among OSB27SJ, OSB27BJ, OSB27SM, and OSB27BM were calculated based on the NRT2:1 gene sequences using MEGA in order to compare community differences to changes in environmental parameters. While no environmental parameter showed a statistically significant relationship with diatom community differences, ammonia and temperature showed an identical R^2 value of 0.23. This implies that temperature has as much an effect on changes in diatom community as ammonia does, perhaps explaining why greater diversity and clustering were seen among seasons when temperature gradients were highest.

T-RFLP analysis with functional genes instead of 18S rRNA genes allowed for the observation of a specific class of organism (diatoms) in a given environment instead of all species present. This is why the functional gene NRT2:1 was chosen to examine changes in diatom communities in Onslow Bay. The presence or absence of T-RF peaks in a given sample can be used to compare changes in diatom communities. In this study, the community differences were calculated by hand for all 18 samples from OSB and compared to environmental data. The results indicated that temperature had more influence on diatom community changes than nitrogen levels. This would be compatible with the results seen in the sequence analysis.

CONCLUSION

Various factors could influence the structure and productivity of diatom communities. In order to understand community responses to environmental variations, molecular tools targeting diatom NRT2:1 genes were developed and applied to examine diatoms communities separate from all other phytoplankton in Onslow Bay. Inorganic nutrients such as ammonium and nitrate were expected to govern the changes in diatom communities, but nitrogen levels showed less of an effect on community variation than temperature. Diversity of diatom communities was decreased as temperature was raised in May. However, temperature would not be the only driving force behind diatom community change. Further studies should follow to examine other environmental parameters such as light intensity, silica, metals, and salinity on diatom community dynamics.

CHAPTER 3: MONITORING DIATOM COMMUNITY DYNAMICS IN MONTEREY BAY, CA, USING THE NITRATE TRANSPORTER GENE AS A GENETIC MARKER

INTRODUCTION

Monterey Bay, located at the edge of the California Current, comprises the eastern boundary of the North Pacific gyre. It is a deep embayment that has regular, predictable periods of upwelling when cold, nutrient-rich water is brought to the surface. These upwelling conditions support high levels of phytoplankton productivity that have been studied for over 50 years (Wilkerson et al. 2000). Diatoms, primarily *Chaetocerous* and *Nitschia* spp., make up nearly 50% of the phytoplankton community in Monterey Bay (Kudela and Dugdale 2000) and dominate the phytoplankton community in Monterey Bay nearly 100-fold according to carbon content (Wilkerson et al. 2000). Oceanic phytoplankton perform nearly 50% of the global fixation of carbon (Field et al. 1998), which is estimated to be as much atmospheric carbon fixation per year in primary production as terrestrial rain forests (Parker and Armbrust 2005). Since diatoms make up a large majority of the oceanic phytoplankton community, diatom communities have significant roles in marine primary production and global primary production.

Diatoms are influenced by available nutrients, especially nitrogen and silica (Kudela and Dugdale 2000). The increase of diatom biomass measured off the coast of Monterey Bay was linked to increases in nitrate concentrations during upwelling (Wilkerson et al. 2000). Diatom biomass increased 1.5 to 7-fold when natural assemblages from the Long Island Sound, NY, were incubated with nitrogen addition (Gobler et al. 2006). The increases of diatom biomass (12-26 %) were also observed in

nitrogen amendment experiments with samples from the Pamlico Sound, NC (Piehler et al. 2004).

Silica is an important nutrient for diatom growth due to the formation of a silica shell around diatom cells. In mesocosm experiments in which natural phytoplankton assemblages consisting of 50% diatoms were enriched with both silica and nitrate, nitrate uptake rates were influenced by silica levels. The concentration of silica adjusted the rate at which nitrogen limitation occurred, but silica levels did not directly control biomass accumulation (Kudela and Dugdale 2000).

Nitrogen in Monterey Bay is supplied by a variety of sources. The primary form of nitrogen is nitrate that is upwelled during the spring (Wilkerson et al. 2000). Nitrification carried out by bacteria in suboxic bottom waters releases nitrate that is brought to the surface during upwelling. Ammonia from decomposition also plays a part in the nitrogen cycle in Monterey Bay, although it does not support as much production as upwelled nitrate does (Wilkerson et al. 2000).

The purposes of this study were to examine the composition and dynamics of diatom communities in Monterey Bay, CA, over different seasons and depth profiles using the nitrate transporter gene as a genetic marker and to determine potential links between environmental parameters and diatom community variation. The sequences and T-RFLP fingerprints of diatom NRT2:1 genes will be compared to temperature, nitrate, and ammonium levels to determine their correlations.

MATERIALS AND METHODS

Samples and DNA Extraction.

Water samples from the surface (10 m) and mid depth (30 m) of Monterey Bay were collected during May and October, 1998. Approximately four liters from each sample were filtered on 0.2-μm-pore-sized supur filters (Gelman) by tangential flow filter cassettes (Ultrasette 300 KD open channel; Filtron Technology Crop., Northborough, Mass.) to collect phytoplankton cells. The filters were labeled as GS21 and GS22 for the sample collected from the surface and mid-depth, respectively, in May, when upwelling occurred. The filters with GS51 and GS52 were from the samples collected from the surface and mid-depth in October when nutrients were depleted (Figure 11). All the filters were stored at -80^{o}C until processing. DNA was extracted from the filters using a phenol-chloroform extraction according to Ausubel et al. (1999) and resuspended in 100 μl of sterile water.

At each sampling time, temperature was measured using a Seabird CTD sensor. Nitrate and silica were measured either manually using a colorimetric technique (Strickland and Parsons 1972) or using a Lachat autoanalyzer (Table 6).

Figure 11. Map of Monterey Bay, with sampling site marked in white.

Table 6. Environmental parameters measured in Monterey Bay.

Name	Temp (^{o}C)	NO_3 (μM/L)	Si (μM/L)
GS51 (Surface, Oct.)	13.36	6.31	6.14
GS52 (Mid, Oct.)	11.48	19.09	17.05
GS21 (Surface, May)	11.7	14	16.78
GS22 (Mid, May)	9.56	27.52	30.52

PCR Amplification of Nitrate Transporter Genes

In order to examine diatom community structure in Monterey Bay, high affinity nitrate transporter (NRT2:1) genes were amplified with specific primers designed by Song and Ward (2007) (Table 3). Nested PCR amplification using the primers DANAT1F and DANAT1R in the first reaction and the primers DANAT3F and DANAT2R in the second reaction was conducted with DNA extracted from samples collected from GS21, GS22, GS51, and GS52. In both reactions the 25 μL reaction mix contained 1 μL template DNA, 200 nM each primer, 0.5 μL *Taq* polymerase, 50 μM each deoxynucleotide triphosphate, 2 mM $MgCl_2$, and buffer (500mM KCl, 200mM Tris-HCl [ph 8.4]). The template for the initial PCR reaction was DNA extracted from the environment, and the PCR mixtures of the initial reaction were used as a template for the second reaction. The PCR cycle started with an initial denaturation at 95°C for 10 minutes, and 30 cycles of PCR were as follows: 30 seconds at 95°C, 30 seconds at 55°C, 1 minute at 72°C. After the last cycle, the reaction was extended at 72°C for 10 minutes. PCR products were verified by electrophoresis with 1% agarose gel.

Cloning and Sequencing of NRT2:1 genes.

The amplified products were gene cleaned using the PerfectPrep Gel Clean Up Kit (Promega, Madison, WI). A total of 2μL of purified amplicon was used in cloning using a TOPO TA Cloning® Kit for Sequencing (Invitrogen Corporation, Carlsbad, CA) using half the volumes specified by the manufacturer. The ligated plasmids were

transformed in high-transforming-efficiency *Escherichia coli* TOP10® cells (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's protocol and then plated on Lauria Broth agar plates containing 10µg/mL of kanamycin and 100mg/mL of X-gal (5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside). The positive clones carrying the amplified products were determined on the basis of white and blue color screening. The positive clones were picked with sterile toothpicks and grown in LB broth containing10µg/mL of kanamyacin in a 96-well plate. Clonal libraries of NRT2:1 genes were generated in a 96- well plate for each amplified product from GS21, GS22, GS51, and GS52.

Approximately 30 clones from each library were screened for inserts using colony check PCR with the M13R and T7 primers according to the following protocol: the 25 µL reaction mix contained 1 µL cloned cells, 200 nM each primer, 0.5 µL *Taq* polymerase, 50 µM each deoxynucleotide triphosphate, 2 mM $MgCl_2$, and buffer (500mM KCl, 200mM Tris-HCl [ph 8.4]). After an initial denaturation at 95^{o}C for 10 minutes, the PCR parameters for 30 cycles were as follows: 30 seconds at 95^{o}C, 30 seconds at 55^{o}C, 1 minute at 72^{o}C. After the last cycle, the reaction was extended at 72^{o}C for 10 minutes. PCR products were then subjected to electrophoresis with 1% agarose gel.

Approximately 30 positive clones from each library were sequenced. Aliquots (1 µL) of products from the colony screening PCR was used for each sequencing reaction, along with either the primers M13R or T7. DNA sequencing was performed with an ABI PRISM BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Samples were initially denatured by heating to a temperature of 96^{o}C at a rate of

1°C/second and then holding at 96°C for 1 minute. After the initial denaturing, the PCR parameters for 25 cycles were as follows: cooling to a temperature of 50°C at the rate of 1°C/second, holding at 50°C for 5 seconds, heating to 60°C at the rate of 1°C/second, holding at 60°C for 4 minutes, heating to 96°C at the rate of 1°C/second, holding at 96°C for 10 seconds. After the last cycle, the samples were held at 50°C for 5 seconds and then 60°C for 4 minutes. An ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA) was used to analyze the samples, and DNA sequences were assembled and edited using the Sequencher program, version 4.0 (Gene Codes Corporation). A neighbor-joining tree was produced from the sequence data using the MEGA software version 3.1 (Kumar et al. 2004) using the Kimura 2-parameter model for nucleotide substitutions and 500 bootstrap replicates.

T-RFLP Analysis of NRT2:1 Genes

A nested PCR amplification was performed as described above using the primers DANAT1F and DANAT1R in the first reaction and the fluorescently-labeled primer DANAT3F(6'-FAM) and DANAT2R in the second reaction. PCR products were subjected to electrophoresis in 1% agarose gel and the band of the correct size excised and extracted using the PerfectPrep Gel Clean Up Kit (Promega, Madison, WI). A total of 10 μL of purified PCR products were digested overnight with 0.5 U of *HaeIII* enzyme in 15 μL at 37°C. The digested product was precipitated with 75% isopropanol and resuspended in 10 μL of Hi-Di formamide with GS500 ROX size standard (Applied Biosystems, Foster City, CA) and then denatured for 90 seconds at 95°C and placed

immediately on ice for 1 minute. The digested products were run on an ABI 3130xl genetic analyzer (Applied Biosystems, Foster City, CA) in GeneScan mode. T-RF fragments with more than 10% of the height of the largest T-RF were considered for further analysis (Figure 2).

Community Structure Analysis

Community structures of diatoms in Monterey Bay were compared with sequences from the NRT2:1 gene libraries and T-RFLP fingerprints. Pairwise comparisons of T-RFLP fingerprints were conducted for each sample, and a community diversity index (C) for each comparison was calculated using the formula: 1 - (W/(a1+a2)) where W is the number of peaks samples 1 and 2 have in common, a1 is the total number of peaks in sample 1, and a2 is the total number of peaks in sample 2 (modified from Hewson and Fuhrman 2004). Pairwise comparisons of each environmental parameter (temperature, nitrate and silica) for each sample were also conducted by noting the change in each parameter between each sample.

Simple linear regression was performed for the combined data set of all 4 samples against each environmental parameter using Microsoft Excel. Canonical Correspondence Analysis (CCA) was also conducted on all 4 samples using the PC-ORD version 4.20 software (McCune and Mefford 1999). Community differences based on the presence or absence of peaks of given sizes were determined using Jaccard's index for all analysis, and community differences were compared to the differences in temperature,

nitrate and silica levels. Statistical significance was determined using a Monte Carlo test and Mantel's test.

Sequences of the NRT2:1 genes from all four samples were used to determine the variation in diatom communities using MEGA version 3.1 (Kumar et al. 2004). Sequences were aligned in MEGA and obvious errors were amended. Sequences were designated into four groups based on their clonal libraries: GS21, GS22, GS51, and GS52. Net community difference indices were then calculated as net between group means using the Kimira-2 parameter model. The net community difference indices were compared to differences in environmental parameters and simple linear regression was performed for each environmental parameter using Microsoft Excel.

Diatom community diversity was compared using DOTUR to investigate patterns of diversity over time and space. A rarefaction curve was produced using DOTUR (Schloss and Handelsman 2005) and a cutoff of 4% differences in sequences were used to determine the operational taxonomic units (OTUs) for the OSB27SJ, BJ, SM, and BM libraries. Chao[1] estimator and Shannon indices were also used to determine diversity of the NRT2:1 genes in Monterey Bay.

RESULTS

PCR Detection of NRT2:1 Gene

A nested PCR was performed with DNA extracted from Monterey Bay samples using primers specific for diatom nitrate transporter genes (NRT2:1). No product was

observed from the initial reaction. The expected 750 bp product was obtained from all samples after nested PCR was performed.

The amplified NRT2:1 genes from GS21, GS22, GS51, and GS52 were cloned and sequenced. The number of sequenced clones from each library is listed in Table 7.

Phylogenetic Analysis of NRT2:1 Genes

All the sequences from the clones were determined to be putative diatom NRT2:1 genes based on Blastx analysis. Phylogenetic analysis clustered most of the sequences in 20 different groups, except for 8 sequences (Figure 12). Clusters A, D, I, J, N, O, R and T contained the sequences from two different libraries. Clusters D, I and J contained the sequences from the surface and mid-depth of May, which might represent the diatom populations upwelled and mixed in this month. Most of the clones from the surface and mid-depth of May (GS21 and GS22), and the mid-depth of October (GS52) formed unique clusters with the sequences from the same samples. However, only 4 unique sequences were found in the sample collected from the surface of October (GS51) and most of the sequences were associated with those from the GS52 library. This implies that diatom communities in two different depths in October are more similar than those in May. Seasonal variation in diatom communities was clearly demonstrated based on the NRT2:1 gene sequences.

More than 50% of the NRT2:1 gene clones (58 clones) were somewhat related to those found in reference diatom species. The sequences in clusters C and D shared more

Table 7. PCR detection of NRT2:1 and number of positive clones sequenced from Monterey Bay.

Sample	DANAT 1F/DANAT 1R	DANAT 3F/DANAT 2R	# of clones
GS21	-	+	34
GS22	-	+	31
GS51	-	+	33
GS52	-	+	36

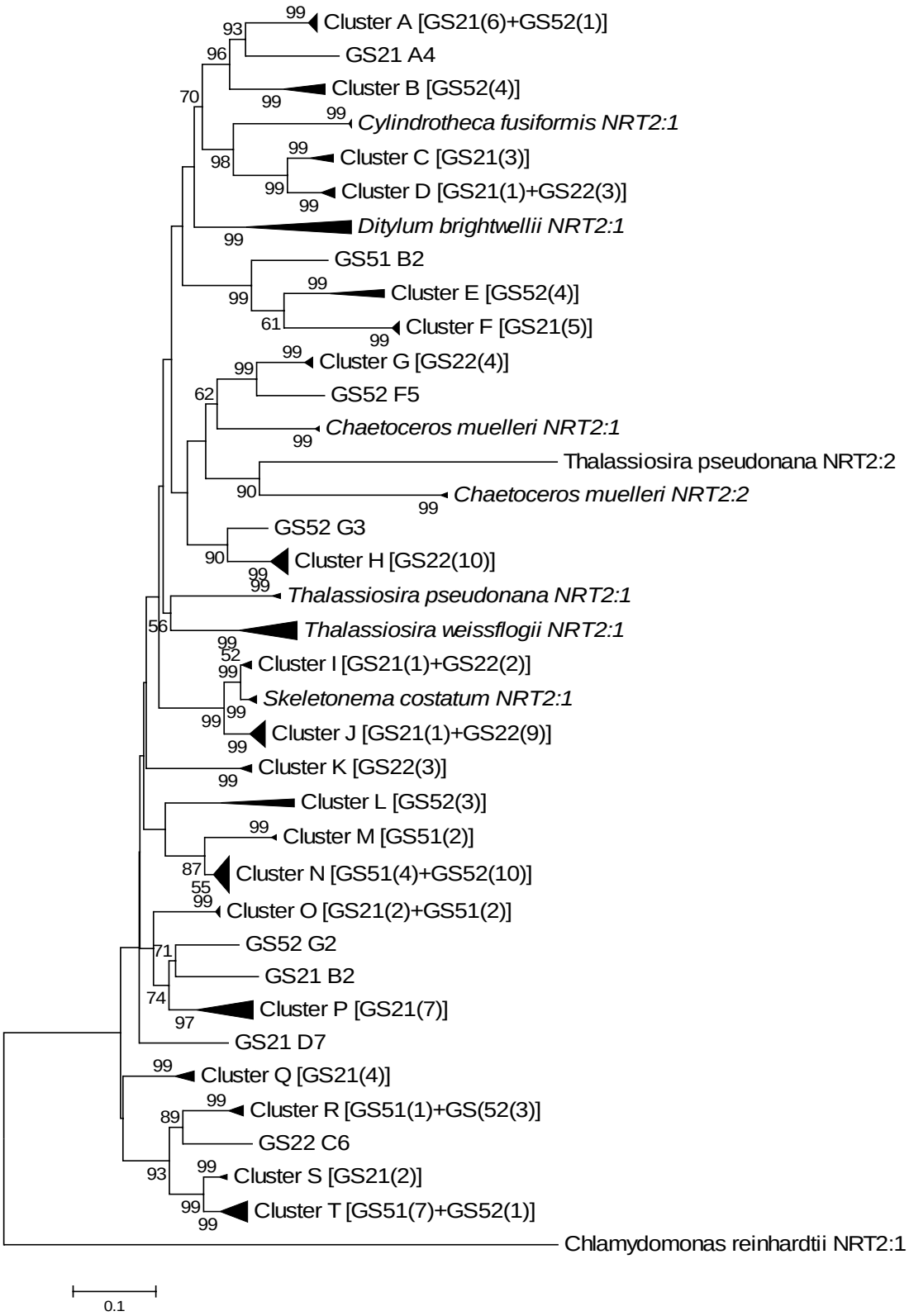

Figure 12. Phylogenetic tree of NRT2:1 genes from Monterey Bay.

than 65% similarities to those in *C. fusiformis*. Clusters G and H and clones GS52 F5 and GS52 G3 had 60% sequence similarities to the NRT2:1 genes in *Chaetoceros muelleri*. The sequences in clusters I and J were closely associated with those of *Skeletonema costatum*. It appeared that diatoms blooming in the upwelling season (May) were closely related to cultivated diatom species.

Community Structure Analysis Based on the NRT2:1 Gene Sequences

A rarefaction analysis of the NRT2:1 genes from the GS21, GS22, GS51, and GS52 libraries was conducted to determine phylotype richness of diatom communities in Monterey Bay (Figure 13). OTUs represent the number of distinct phylotypes present in a given community. The highest richness was found in October samples (GS51 and GS52) with 19 OTUs, while communities in the surface in May (GS21) had 16 OTUs, and the community in the mid depth of May (GS22) had 8 OTUs.

Chao1 and Shannon indices were also used to measure phylotype richness and diversity of the NRT2:1 genes (Table 8). The Chao1 index, which is a nonparametric estimator, gives a minimum estimation of species richness. October samples have a Chao1 estimate of 38.5 and 40.5 OTUs, indicating higher phylotype diversity than May samples (25 and 8.5 OTUs). The Shannon index is an estimation of species richness and diversity that ranges from 0 to about 4.5, with the higher range representing the most species diversity. The Shannon index also indicates that October samples (2.67 and 2.62) have higher phylotype diversity than May samples (2.50 and 1.81). Surface samples tended to have higher phylotype diversity than mid-depth samples.

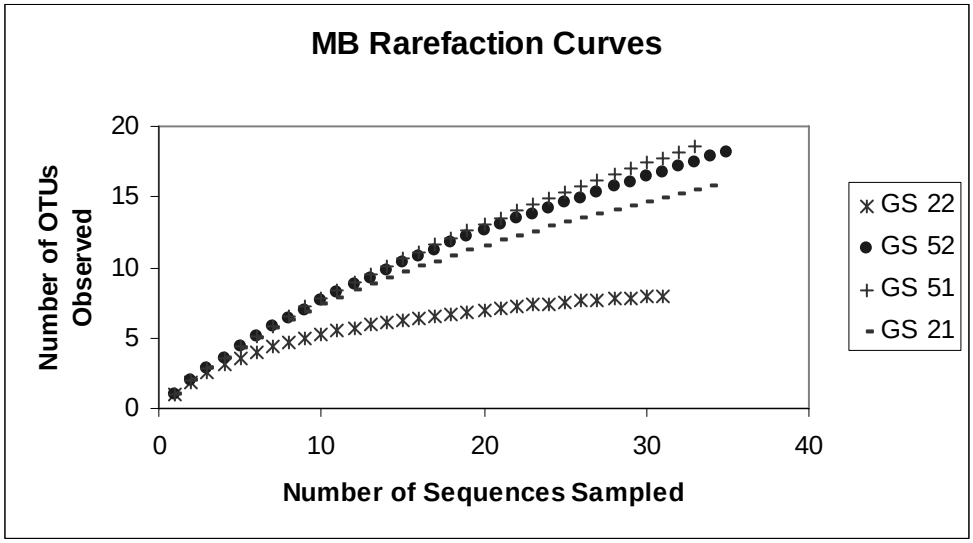

Figure 13. Rarefaction analysis of Monterey Bay clone libraries

Table 8. Chao1 and Shannon indices of diversity for Monterey Bay libraries.

Site	Sequence #	Rarefaction #	Chao1 estimator	Shannon index
GS21	35	16	25	2.49812
GS22	34	8	8.5	1.8072
GS51	32	19	38.5	2.66979
GS52	36	18.5	40.5	2.62603

MEGA analysis was conducted to compare net community differences of the NRT2:1 sequences to environmental parameters and to investigate potential links between environmental parameters and diatom community variation (Figure 14). The community differences based on the MEGA analysis ranged from 0.023 to 0.089 (Table 9) with GS22 and GS51 having the highest diversity and GS51 and GS52 having the lowest. There was no statistically significant correlation between the differences of environmental parameters and diatom communities (all R^2 values <0.67).

T-RFLP Analysis and CCA

Spatial and temporal variation of marine diatom communities in Monterey Bay were examined using T-RFLP analysis of the NRT2:1 genes (Figure 15). T-RFLP peaks with heights greater than 10% of the highest peak were considered for analysis as separate from the background. T-RF peak sizes ranged from 51 to 691 bp, and each sample contained between 6 and 11 peaks. All four samples contained a T-RF peak of 175 bp. The T-RFs were unable to be identified in the sequenced clones based on *in silico* analysis.

Pairwise comparisons between T-RFLP fingerprints and environmental variations showed no correlation. Silica showed the highest correlation with an R^2 value of 0.17 (data not shown). The presence or absence of T-RF peaks in T-RFLP can also be converted into a binary sequence and used in CCA. CCA is a statistical analysis specifically designed to relate environmental variation to community variation. CCA was conducted with T-RFLP fingerprints as compared to the concentration of nitrate and

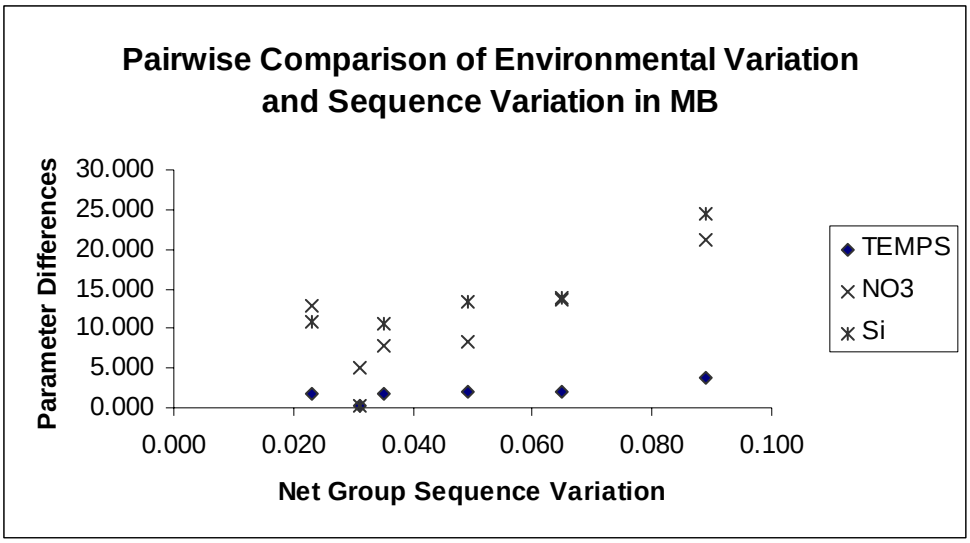

Figure 14. Pairwise comparison of environmental variation and NRT2:1 gene sequences in Monterey Bay.

Table 9. Net community differences based on MEGA analysis

	GS 21	**GS 22**	**GS 51**
GS 21			
GS 22	0.07		
GS 51	0.04	0.09	
GS 52	0.03	0.05	0.02

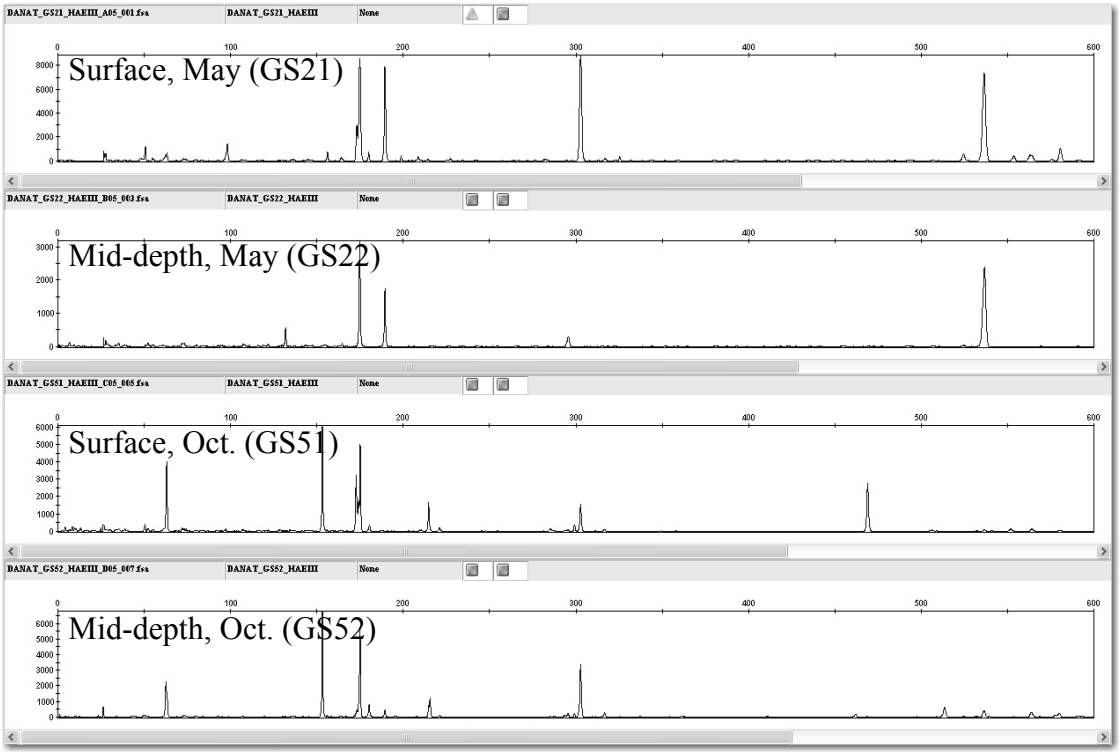

Figure 15. T-RFLP fingerprints of diatom NRT2:1 genes from Monterey Bay.

silica, and temperature (Figure 16). The communities were roughly separated into two groups depending on the season. Temperature and nitrate/silica were negatively correlated to each other at nearly a 1:1 ratio. This result makes it difficult to differentiate the main factor influencing diatom community changes, but all three parameters could affect the variation in the composition of diatom assemblages in Monterey Bay.

Mantel's test to determine correlation between two distance matrices was also performed using all T-RFLP fingerprints and environmental data (nitrate, silica, and temperature). No correlation was found ($p=0.164$), but this result could be biased due to the small number of samples.

DISCUSSION

It was hypothesized that changes in diatom communities would be most influenced by changes in nutrient regimes, specifically nitrogen levels, because of the extreme amounts of nitrogen brought into the photic zone by upwelling during the spring. However, upwelling also affects the temperature of the water, with colder water being pulled to the surface. The general pattern of clustering seen in the phylogenetic tree may reflect the change in nutrient or temperature levels from season to season.

DOTUR analysis was used to examine the diversity of the diatom communities in Monterey Bay. Phylotype diversity and richness based on the Chao1 and Shannon indices were the greatest in October when no upwelling events occurred. During non-upwelling seasons nutrient levels were lower and temperature was higher than during

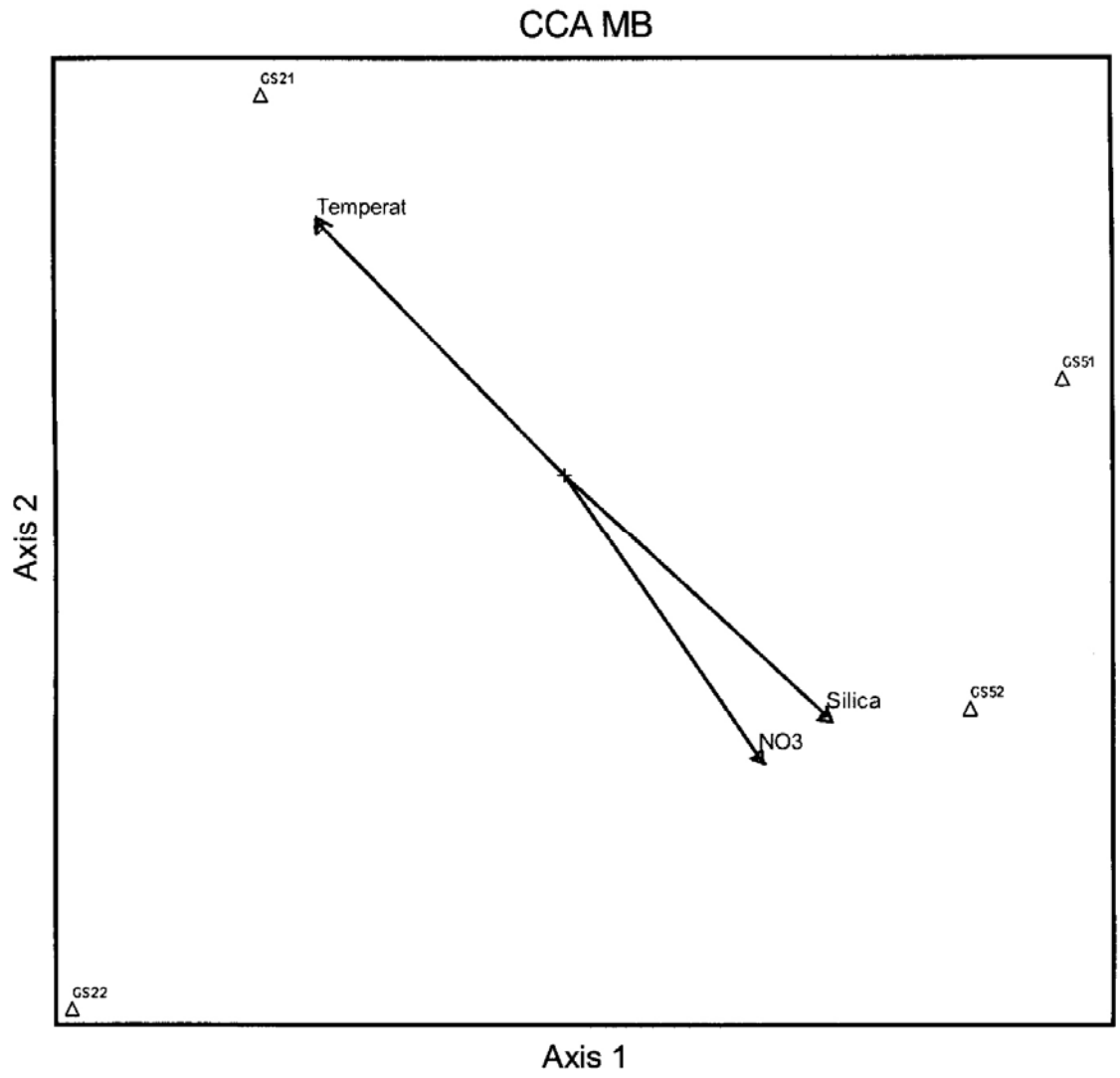

Figure 16. CCA of Monterey Bay based on T-RFLP fingerprints and environmental parameters.

upwelling. Diatom blooms occurred corresponding to upwelling events and were usually dominated by a few species of diatoms (Wilkerson et al. 2000), which could lead to lower diversity of diatoms in phytoplankton communities.

The net community differences between GS21, GS22, GS51, and GS52 samples were calculated using MEGA and compared to the changes in environmental parameters. Silica and temperature showed a correlation with the community changes with R^2 value of 0.67 and 0.65, respectively. However, there was no statistical significance in this correlation. This implies that temperature and silica appeared to have more influence on changes in diatom community than nitrate. This finding was also supported by T-RFLP analysis of the NRT2:1 genes.

CCA with T-RFLP fingerprints showed interesting trends although no statistically significant relationships were detected between community structure and environmental parameters. Temperature and nutrients (silica and nitrate) had an almost directly inverse relationship (correlation = -0.999). This implies that nitrate and silica vary with temperature, which makes it difficult to determine whether nutrients or temperature is more influential on diatom communities.

CONCLUSION

Environmental influences on diatom communities are numerous and complex. By targeting diatom NRT2:1 genes, diatom communities were examined separately from all other phytoplankton present in Monterey Bay. Nitrogen level variation was expected to have the greatest influence on diatom community change due to the involvement of

upwelling events in Monterey Bay. However, nitrogen levels appeared to have much less effect on diatom community variations than temperature and the levels of silica. It is unclear how temperature affects the levels of nutrients in water. Further studies could examine the interactions of temperature and nutrient levels as well as investigate the controlling effects of other environmental parameters such as light intensity, ammonia, trace metals, and salinity on diatom community dynamics.

CHAPTER 4: COMPARISON OF DIATOM COMMUNITIES IN ONSLOW BAY, NC AND MONTEREY BAY, CA

Diatoms make up the dominant phytoplankton communities in Monterey Bay, CA, and Onslow Bay, NC, even though both bays have very different nutrient regimes. Monterey Bay is characterized by upwelling events that cause an increase in nutrient levels in the water column and diatom blooms in the spring, while lower levels of diatom biomass are found during non-upwelling seasons. In contrast, Onslow Bay has a similar spring bloom but with no upwelling event and a less pronounced increase in nutrients. Since diatoms make up a large majority of the phytoplankton community in these bays, diatom communities have significant roles in primary production in both areas. This study was conducted to investigate the environmental parameters that influence the changes in diatom communities in both bays. Molecular analyses of NRT2:1 genes were used to examine diatom communities under varying conditions of environmental parameters. It was hypothesized that changes in nitrogen would show the biggest influence on changes in diatoms.

PHYLOGENETIC ANALYSIS OF NRT2:1 GENES

All the sequences from the clones were determined to be putative diatom NRT2:1 genes based on Blastx analysis. Phylogenetic analysis showed all the sequenced clones were more closely related to the NRT2:1 genes found in diatoms than to a green alga *Chlamydomonas reinhardtii* (Figure 17). Phylogenetic analysis clustered most of the sequences in 23 different groups, except for 13 sequences. Clusters A, B, C, E, L, R,

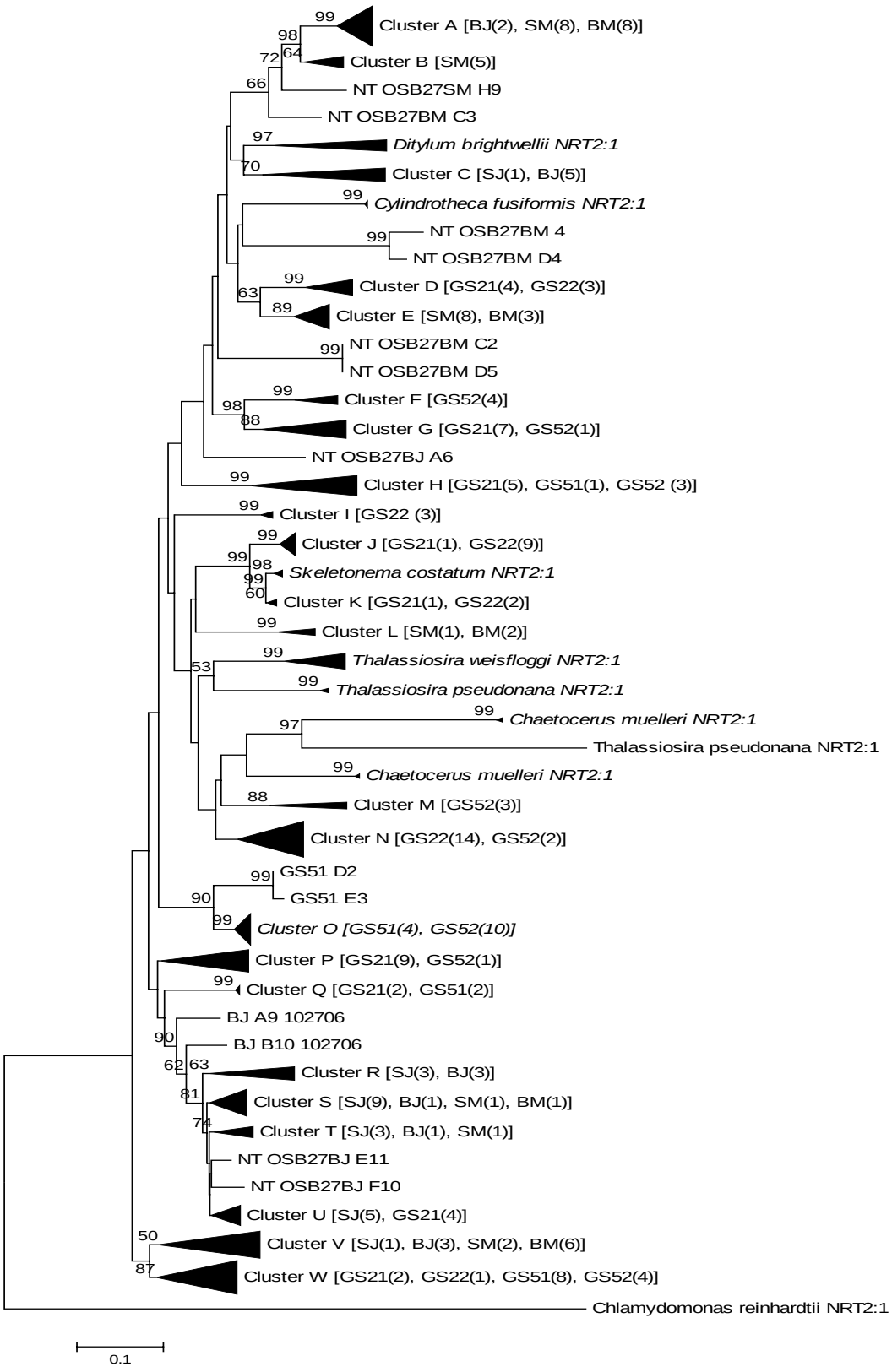

Figure 17. Phylogenetic tree of NRT2:1 genes from Onslow Bay and Monterey Bay.

S, T, and V contain sequences only found in Onslow Bay, while clusters D, F, G, H, I, J, K, M, N, O, P, Q, and W contain sequences only found in Monterey Bay. Only cluster U contained sequences from both bays, indicating unique populations in each bay. Several unique populations of diatoms were present at each sample as shown in cluster B, F, I, and M. Clusters C, D, E, J, K, L, O, and R contained sequences from the surface and bottom of a single month, which might represent the diatom populations upwelled and mixed in these months. Only four clusters (G, N, P, and Q) contained sequences found in different months. Seasonal and spatial variation in diatom communities was clearly demonstrated based on the NRT2:1 gene sequences.

Two sequences from the Onslow Bay bottom May sample (OSB BM4 and OSB BM D4) were related to the genes found in the pinnate diatom *Cylindrotheca fusiformis* with more than 55% sequence similarities (Figure 17). Cluster C, containing six NRT2:1 genes from Onslow Bay January libraries, was related to the genes found in *Ditylum brightwellii* with more than 64% sequence similarities. The three NRT2:1 sequences from Monterey Bay collected in May that grouped in cluster K were related to the genes found in *Skeletonema costatum* with more than 95% sequence similarities. Three NRT2:1 sequences collected from the bottom of Monterey Bay in October (cluster M) were related to the genes found in *Chaetocerous muelleri* with more than 67% sequence similarities. However, most of the NRT2:1 genes found in Onslow Bay and Monterey Bay were not closely related to the genes found in the reference diatom species.

Phylogenetic analysis of the putative diatom NRT2:1 genes from Monterey Bay and Onslow Bay shows spatial and seasonal separation between the diatom communities

of Onslow Bay and Monterey Bay. Therefore, both seasonal and spatial variation in diatom communities found in Onslow Bay and Monterey Bay was clearly demonstrated based on the NRT2:1 gene sequences.

COMMUNITY STRUCTURE ANALYSIS BASED ON THE NRT2:1 GENE SEQUENCES

A rarefaction analysis of the NRT2:1 genes from the Onslow Bay OSB27SJ, BJ, SM, and BM libraries and the Monterey Bay GS21, GS22, GS51, and GS52 libraries was conducted to compare the diversity of diatom communities in the two bays (Figure 18). Using a 4% difference as a definition of a phylotype of the NRT2:1 gene sequences, the highest richness was found in Onslow Bay in January (21 and 20 OTUs), with Monterey Bay October samples (GS51 and GS52) having the next highest richness (19 OTUs). Communities from the surface in May in both bays had 16 OTUs, and the community in the mid depth of May in Monterey Bay (GS22) had the lowest richness with 8 OTUs.

The sequences were further examined using the Chao1 and Shannon indices to determine phylotype richness and diversity, respectively. Both indices were calculated using a 4% difference definition of a phylotype (Table 10). January samples in Onslow Bay have a Chao1 estimate of 87 and 96.5, indicating higher phylotype diversity than May samples in Onslow Bay (29.75 and 38) and all samples collected from Monterey Bay (Table 10). The Shannon index also indicates that January samples from Onslow Bay (2.88 and 2.93) have higher phylotype diversity than May samples in Onslow Bay

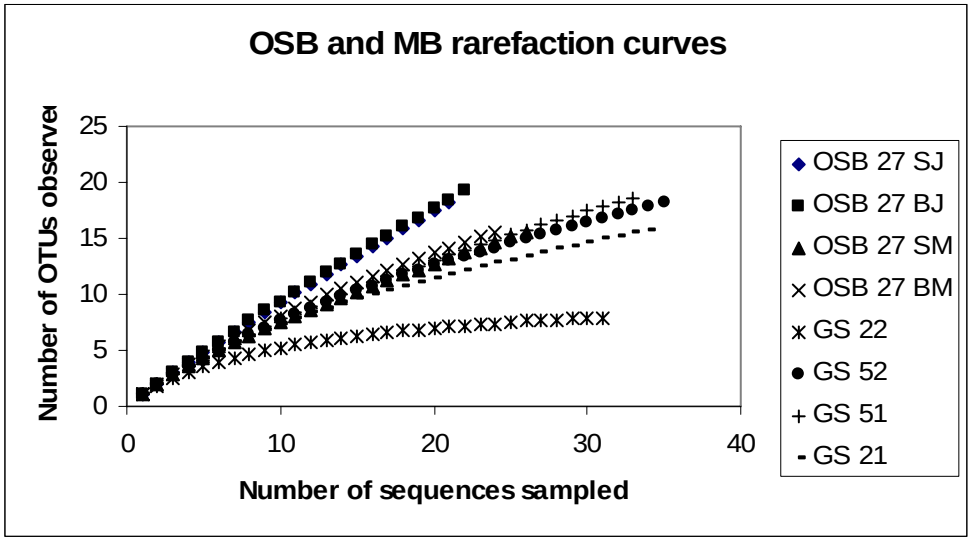

Figure 18. Rarefaction analysis of Onslow Bay and Monterey Bay clone libraries.

Table 10. Chao1 and Shannon indices of diversity for Monterey Bay and Onslow Bay libraries.

Site	Sequence #	Rarefaction #	Chao1 index	Shannon index
OSB27SJ	22	21	87	2.87822
OSB27BJ	23	20	96.5	2.93192
OSB27SM	27	16	38	2.49694
OSB27BM	25	17	29.75	2.5988
GS21	35	16	25	2.49812
GS22	34	8	8.5	1.8072
GS51	32	19	38.5	2.66979
GS52	36	18.5	40.5	2.62603

(2.50 and 2.60) and any sample from Monterey Bay. Higher diversity in Onslow Bay may be due to high biomass blooms during upwelling in Monterey Bay being dominated by only a few diatom species, reducing the overall level of diversity in Monterey Bay.

CONCLUSION

This study indicated that, according to all molecular analyses, temperature seemed to have a greater influence on diatom communities in both Monterey Bay and Onslow Bay, but nutrient levels also influenced the diatom communities in Monterey Bay. Interestingly, nitrogen levels seemed to have the least influence on diatom communities in both bays. In Onslow Bay this may be because nitrate stayed at an extremely low level year-round, reaching only 4.4 μM/L at its highest point. In Monterey Bay, nitrate levels were negatively correlated almost 1:1 with temperature. It is impossible to say whether the change in temperature or the change in nitrate more heavily influenced the change in the diatom community using the analysis in this study; and the influence of nitrate, silica, and temperature all had very similar R^2 values for Monterey Bay.

The influences of community change in marine diatoms are very complex. Other nutrients that this study did not examine certainly play a part in determining changes in diatom communities. It is most likely that interactions of physical parameters and nutrients have the greatest influence in diatom community dynamics.

Monitoring variations in the environment over temporal and spatial distances will promote understanding of forces behind changes in diatom communities. Investigations

of diatom community dynamics could shed light on changes in diatom physiology that allow for shifts in diatom communities, which may also be applicable to other types of phytoplankton. Some diatom species, such as *Chaetocerous* spp. and *Pseudo-nitzchia* spp., can be harmful in large numbers. Monitoring diatom communities at different seasons and locations can promote understanding of what can cause shifts in diatom community composition or production, which will assist in predicting the onset and extent of harmful diatoms' effects on the rest of the environment. Finally, diatoms are the dominant primary producer in the world's marine environments, and tracking changes in diatom communities between seasons and locations can help us track periods of increased or decreased primary production and subsequent changes in secondary production. Understanding the factors that influence diatom community dynamics is an important aspect of understanding the foundation of the global marine ecosystem.

LITERATURE CITED

Agawin N. S. R., C. M. Duarte, S. Agusti. 2000. Nutrient and temperature control of the contribution of picoplankton to phytoplankton biomass and production. Limnol. Oceanogr. 45(3): 591-600.

Allen A. E., B. B. Ward, B. Song. 2005. Characterization of diatom (Bacillariophyceae) nitrate reductase genes and their detection in marine phytoplankton communities. J. Phycol. 41: 95-104.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.). 1999. Short protocols in molecular biology. John Wiley and Sons, New York, NY.

Berges J. A. 1997. Algal nitrate reductases. Eur. J. Phycol. 32: 3-8.

Berges J. A., and P. J. Harrison. 1995. Nitrate reductase activity quantitatively predicts the rate of nitrate incorporation under steady state light limitation: a revised assay and characterization of the enzyme in three species of marine phytoplankton. Limnol. Oceanogr. 40(1): 82-93.

Bouman H., T. Platt, S. Sathyendranath, V. Stuart. 2005. Dependence of light-saturated photosynthesis on temperature and community structure. Deep-Sea Research I 52: 1284-1299.

Braker, G., J. Zhou, L. Wu, A. H. Devol, J. M. Tiedje. 2000. Nitrite reductase genes (*nir*K and *nir*S) as functional markers to investigate diversity of denitrifying bacteria in Pacific Northwest marine sediment communities. Appl. Env. Microbiol. 66 (5): 2096-2104.

Braker, G., H. L. Ayala-del-Rio, A. H. Devol, A. Fesefeldt, J. M. Tiedje. 2001. Community structure of denitrifies, *Bacteria*, and *Archaea* along redox gradients in Pacific Northwest marine sediments by terminal restriction fragment length polymorphism analysis of amplified nitrite reductase (*nir*S) and 16S rRNA genes. Appl. Env. Microbiol. 67 (4): 1893-1901.

Cahoon, L. B., and R. A. Laws. 1993. Benthic diatoms from the North Carolina continental shelf: inner and mid shelf. J. Phycol. 29: 257-263.

Campbell W. H. 1999. Nitrate reductase structure, function and regulation: bridging the gap between biochemistry and physiology. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50: 277-303.

Conway H. L. 1977. Interactions of inorganic nitrogen in the uptake and assimilation by marine phytoplankton. Marine Biology 39: 221-232.

Collos Y., A. Vaquer, P. Souchu. 2005. Acclimation of nitrate uptake by phytoplankton to high substrate levels. J. Phycol. 41: 466-478.

Crawford N. M., and A. D. M. Glass. 1998. Molecular and physiological aspects of nitrate uptake in plants. Trends in Plant Science 3(10): 389-395.

Dortch, Q. 1990. The interaction between ammonium and nitrate uptake in phytoplankton. Mar. Ecol. Prog. Ser. 61: 183-201.

Egge, J. K., and D. L. Aksnes. 1992. Silicate as regulating nutrient in phytoplankton competition. Mar. Ecol. Prog. Ser. 83: 281-289.

Falkowski P. G. 1975. Nitrate uptake in marine phytoplankton: comparison of half-saturation constants from seven species. Limnol. Oceanogr. 20 (3): 412-417.

Field C. B., M. J. Behrenfeld, J. T. Randerson, P. Falkowski. 1998. Primary production of the biosphere: integrating terrestrial and oceanic components. Science 281: 237-240.

Flagg, C. N., L. J. Pietrafesa, and G. L. Weatherly. 2002. Springtime hydrogaphy of the southern Middle Atlantic Bight and the onset of seasonal stratification. Deep-Sea Research II 49: 4297-4329.

Forde B. G. 2000. Nitrate transporters in plants: structure, function, and regulation. Biochimica et Biophysica Acta 1465: 219-235.

Galvan A., and E. Fernandez. 2001. Eukaryotic nitrate and nitrite transporters. Cell. Mol. Life Sci. 58: 225-233.

Gobler, C. J., N. J. Buck, M. E. Sieracki, and S. A. Sanudo-Wilhelmy. 2006. Nitrogen and silicon limitation of phytoplankton communities across an urban estuary: The East River-Long Island Sound system. Estuarine, Coastal and Shelf Science 68: 127-138.

Herrera L., and R. Escribano. 2006. Factors structuring the phytoplankton community in the upwelling site off El Loa River in northern Chile. Journal of Marine Systems 61: 13-38.

Hewson, I., and J. A. Fuhrman. 2004. Richness and diversity of bacterioplankton species
along and estuarine gradient in Moreton Bay, Australia. Applied and Environmental Microbiology 70 (6): 3425-3433.

Hildebrand M., D. R. Higgins, K. Busser, and B. E. Volcani. 1993. Silicon-responsive cDNA clones isolated from the marine diatom *Cylindrotheca fusiformis*. Gene 132: 213-218.

Hildebrand M. and K. Dahlin. 2000. Nitrate transporter genes from the diatom *Cylindrotheca fusiformis* (Bacillariophyceae): mRNA levels controlled by nitrogen source and by the cell cycle. J. Phycol. 36: 702-713.

Karl D. M., R. R. Bidigare, R. M. Letelier. 2001. Long-term changes in plankton community structure and productivity in the North Pacific Subtropical Gyre: the domain shift hypothesis. Deep-Sea Research II 48: 1449-1470.

Kudela R. M., and R. C. Dugdale. 2000. Nutrient regulation of phytoplankton productivity in Monterey Bay, California. Deep-Sea Research II 47: 1023-1053.

Kumar, S., K. Tamura, and M. Nei. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Briefings in Bioinformatics 5: 150-163.

Lohrenz, S. E., D. G. Redalje, P. G. Verity, C. N. Flagg, and K. V. Matulewski. 2002. Primary production on the continental shelf off Cape Hatteras, North Carolina. Deep-Sea Research II 49: 4479-4509.

Lomas M. W., and P. M. Glibert. 1999. Temperature regulation of nitrate uptake: a novel hypothesis about nitrate uptake and reduction in cool-water diatoms. Limnol. Oceanogr. 44(3): 556-572.

Lomas, M. W., and P. M. Glibert. 2000. Comparisons of nitrate uptake, storage, and reduction in marine diatoms and flagellates. J. Phycol. 36: 903-913.

Mallin, M. A., J. M. Burkholder, L. B. Cahoon and M. H. Posey. 2000. North and South Carolina coasts. Marine Pollution Bulletin 41: 56-75.

Mallin, M. A., L. B. Cahoon, and M. J. Durako. 2005. Contrasting food-web support bases for adjoining river-influenced and non-river influenced continental shelf ecosystems. Estuarine, Coastal and Shelf Science 62: 55-62.

Marsh T. L. 1999. Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. Current Opinion in Microbiology 2: 323-327.

McCune, B., and M. J. Mefford. 1999. PC-ORD: Multivariate Analysis of Ecological Data. Version 4.20. MjM Software Design, Gleneden Beach, OR.

Parker, M. S., and E. V. Armbrust. 2005. Synergistic effects of light, temperature, and nitrogen source on transcription of genes for carbon and nitrogen metabolism in the centric diatom *Thalassiosira pseudonana* (Bacillariophyceae). J. Phycol. 41: 1142-1153.

Piehler, M. F., L. J. Twomey, N. S. Hall, and H. W. Paerl. 2004. Impacts of inorganic nutrient enrichment on phytoplankton community structure and function in Pamlico Sound, NC, USA. Estuarine, Coastal, and Shelf Science 61(2): 197-209.

Redalje D. G., S. E. Lohrenz, P. G. Verity, C. N. Flagg. 2002. Phytoplankton dynamics within a discrete water mass of Cape Hatteras, North Carolina: the Lagrangian experiment. Deep-Sea Research II 49: 4511-4531.

Ryther, J. H., and W. M. Dunstan. 1971. Nitrogen, phosphorus, and eutrophication in the coastal marine environment. Science 171: 1008-1013.

Schloss, P. D., and J. Handelsman. 2005. Introducing DOTUR, a computer program for defining operation taxonomic units and estimating species richness. Applied and Environmental Microbiology 71(3): 1501-1506.

Semeneh M., F. Dehairs, M. Elskens, M. E. M. Baumann, E. E. Kopczynska, C. Lancelot,
L. Goeyens. 1998. Nitrogen uptake regime and phytoplankton community structure in the Atlantic and Indian sectors of the Southern Ocean. Journal of Marine Systems 17: 159-177.

Smith G. J., R. C. Zimmerman, R. S. Alberte. 1992. Molecular and physiological responses of diatoms to variable levels of irradiance and nitrogen availability: growth of *Skeletonema costatum* in simulated upwelling conditions. Limnol. Oceanogr. 37 (5): 989-1007.

Song, B., and B. B. Ward. 2007. Molecular cloning and characterization of high-affinity nitrate transporters in marine phytoplankton. J. Phycol. 43: 542-552.

Strickland, J. D. H., and T. R. Parsons. 1972. A practical handbook of seawater analysis, 2nd edition, bulletin 167. Fisheries Research Board of Canada, Ottawa, Canada.

Unkles S. E., D. A. Rouch, Y. Wang, M. Y. Siddiqi, A. D. M. Glass, J. R. Kinghorn. 2004. Two perfectly conserved arginine residues are required for substrate binding in a high-affinity nitrate transporter. PNAS 101 (50): 17549-17554.

Wilkerson, F. P., R. C. Dugdale, R. M. Dudela, F. P. Chavez. 2000. Biomass and productivity in Monterey Bay, California: contribution of the large phytoplankton. Deep-Sea Research II 47: 1003-1022.